Erythropoietin inhibits ferroptosis and ameliorates neurological function after spinal cord injury

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Abstract
Ferroptosis is one of the critical pathological events in spinal cord injury. Erythropoietin has been reported to improve the recovery of spinal cord injury. However, whether ferroptosis is involved in the neuroprotective effects of erythropoietin on spinal cord injury has not been examined. In this study, we established rat models of spinal cord injury by modified Allen’s method and intraperitoneally administered 1000 and 5000 IU/kg erythropoietin once a week for 2 successive weeks. Both low and high doses of erythropoietin promoted recovery of hindlimb function, and the high dose of erythropoietin led to better outcome. High dose of erythropoietin exhibited a stronger suppressive effect on ferroptosis relative to the low dose of erythropoietin. The effects of erythropoietin on inhibiting ferroptosis-related protein expression and restoring mitochondrial morphology were similar to those of Fer-1 (a ferroptosis suppressor), and the effects of erythropoietin were largely diminished by RSL3 (ferroptosis activator). In vitro experiments showed that erythropoietin inhibited RSL3-induced ferroptosis in PC12 cells and increased the expression of xCT and Gpx4. This suggests that xCT and Gpx4 are involved in the neuroprotective effects of erythropoietin on spinal cord injury. Our findings reveal the underlying anti-ferroptosis role of erythropoietin and provide a potential therapeutic strategy for treating spinal cord injury.

Key Words: erythropoietin; ferroptosis; Gpx4; iron overload; lipid peroxidation; mechanism; neurological function recovery; spinal cord injury; spinal neuron; xCT

Introduction
Spinal cord injury (SCI) is a critical condition that can lead to the irreversible loss of sensorimotor functions, thereby drastically reducing the patient quality of life (Dietz and Fouad, 2014). The initial mechanical damage to spinal cord tissue and the secondary injury, including edema, ischemia, inflammatory response and programmed cell death (PCD), make SCI a refractory disorder (Chen et al., 2020; Shi et al., 2021). Increasing experimental evidence has shown that different types of PCD, such as apoptosis, autophagy, necroptosis, pyroptosis and ferroptosis, play essential roles in SCI (Yong et al., 1998; Wang et al., 2014; Lipinski et al., 2015; Zhang et al., 2019; Xu et al., 2021). Therefore, preventing or alleviating PCD might be a promising strategy for clinical treatment of SCI.

Ferroptosis, a novel type of PCD first proposed by Dixon et al. (2012), is characterized by iron overload, elevated mitochondrial membrane density, accumulated lipid peroxidation and glutathione (GSH) depletion (Dixon et al., 2012; Cao et al., 2020; Jhelim and David, 2022). Transferin receptor (Tfr) and ferroportin (Fpn) are the proteins involved in iron uptake and exporter respectively (Hentze et al., 2004; Moos et al., 2007). Ferritin heavy chain (Fth) is the subunit of the Ferritin which is the major iron storage protein (Rui et al., 2021). These proteins are responsible for the maintenance of iron homeostasis. Acyl-CoA synthetase long-chain family member 4 (ACSL4) is the key enzyme of lipid peroxidation and 4-hydroxynonenal (4-Hne) is an end-product of lipid peroxidation (Doll et al., 2017; Hassannia et al., 2019). They can reflect the degree of ferroptosis to a certain extent. Glutamate/cystine reverse transporter system Xc– (xCT) and glutathione peroxidase 4 (Gpx4) are critical to GSH metabolism (Kim et al., 2021). Depletion of xCT and Gpx4 can impair the cellular antioxidant capacity and then lead to ferroptosis (Dixon et al., 2012; Yang et al., 2014). Although ferroptosis was initially defined in cancer cells, recent research has indicated that it is also involved in SCI (Ge et al., 2021, 2022). Bleeding and injury stress contribute to the ferroptosis following SCI. Bleeding caused by contusion is critical in the iron accumulation within the injured spinal cord. Injury stress leads to increased production of reactive oxygen species (ROS) and enhanced excitatory toxicity of glutamate (Chen et al., 2020).

Erythropoietin (EPO) is a cytokine that stimulates the production of erythrocytes. It was primarily used to treat anemia (Wu et al., 1995). With the discovery of EPO receptor in the central nervous system, a neurotherapeutic potential for EPO has emerged (Nagai et al., 2001; Grasso et al., 2005). Several studies confirmed the safety and effectiveness of EPO in treating stroke, epilepsy and Alzheimer’s disease (Larpthaveesarp et al., 2016; Sun et al., 2019; Rosetti et al., 2020; Ureña-Guerrero et al., 2020). Other studies showed that EPO improved the neurological function of paraplegic animals by regulating PCD following SCI (Yamanaka et al., 2018; Zhong et al., 2020). However, the effects of EPO on ferroptosis in SCI have not been studied.

In this study, we hypothesized EPO could promote neurological function recovery after SCI by alleviating neuronal ferroptosis. In our study, we evaluated the neuroprotective effects of EPO in a SCI rat model, determined the changes of ferroptotic biomarkers, and investigated the regulatory effects of EPO on ferroptosis in vivo and vitro.
Methods

Animal model
Previous reports indicated that hematuria may be more pronounced in male rats than in female rats, leading to the choice of females in SCI animal models because of fewer urinary tract complications (Ferrero et al., 2015); therefore, we used female rats in our study. Sprague-Dawley rats (n = 152, female, 150-250 g, 6-8 weeks old) were purchased from the Animal Center of Anhui Medical University (license no. SYXX (Wan) 2017-001) and bred in a standard conditions (12-hour light/dark cycle, temperature: 24 ± 2°C, humidity: 60 ± 5%, pathogen-free and free access to food and water). SCI was induced to intraspinal injection of 2% sodium pentobarbital (2 mL/kg, Beyotime Biotechnology, Shanghai, China). All experiments and procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal protocols were approved by the Ethics Committee of Anhui Medical University on January 21, 2021 (No. LLSC 20201135).

In the first animal experiment, we used two different doses of EPO (Four Rings Biopharmaceutical Co., Ltd., Beijing, China), referring to previous studies (Freitag et al., 2015; Li et al., 2015; Zhong et al., 2020), to treat the SCI rats. Animals were randomized into five groups (n = 5/group): sham (T10 laminectomy with 0.5 mL normal saline), vehicle (SCI with 0.5 mL normal saline), low dose (SCI with 1000 IU/kg EPO), EPO (SCI with 5000 IU/kg EPO), and EPO + RSL3 (SCI with 5000 IU/kg EPO and 100 μM RSL3) groups (Yang et al., 2021; Zhou et al., 2021).

To establish the SCI model, modified Allen’s method was applied (Zhong et al., 2020). T10 laminectomy was performed to expose the spinal cord and the spinal cord was contused by an impactor (10 g weight x 1 cm height). The sham group was subjected to T10 laminectomy only. The rats were intramuscularly injected with penicillin, carprofen (pain relief) for the first 3 consecutive days after the operation.

Saline and drugs were intraperitoneally injected immediately postoperatively and once a week for 2 weeks. Animals were sacrificed at designated time points under anesthesia and injured spinal cords were isolated for further tests. The scheme of animal experiments is shown in Figure 1A.

Behavioral tests
The Basso, Beattie and Bresnahan BBB scale (1995) was used to evaluate the hindlimb motor function (Basso et al., 1995; Wett et al., 2015). The BBB scale ranges from 0 (complete hindlimb disability) to 21 points (normal locomotor function). All rats were scored by two researchers who were blinded to animal groups; analyses were performed before surgery and 1, 3, 7 and 14 days post-injury (dpi). For footprint analysis, we made a track (100 cm long and 10 cm wide) with a darkroom at one end to induce rats to walk straight. Footprints were recorded with red ink and white paper (at the bottom of track). The stride length was measured for quantitative analysis.

Magnetic resonance imaging
Magnetic resonance imaging (MRI) was used to evaluate the intraspinal cord injury. MRI was performed on a 1.5 T MR scanner (Magnetom Aera, Siemens Healthcare, Germany) with a rat whole body coil. Sagittal T2-weighted images were obtained with the following parameters: repetition time = 4079 ms; echo time = 21 ms; field of view = 10 cm × 8 cm; slice thickness = 1.5 mm.

Histological staining
Paraplegic changes in the spinal cords of rats were observed by hematoxylin-eosin (HE) staining. Nissl staining was performed to assess neuron survival, and ferric iron was visualized using Perl’s Prussian blue staining. Rats were anesthetized and sacrificed at 14 dpi. The spinal cord tissues from the injured site were isolated for further analysis. The spinal dura mater and nerve roots were carefully peeled off. The tissues were homogenized in 9-fold (volume/weight) of normal saline. After centrifugation at 600 g for 10 minutes, the supernatant was collected and assayed with a tissue iron assay kit (AO39-2-1) and reduced-GSH assay kit (AO60-2-1). Nanjing Jianghe Biotechnology Co., Ltd., Nanjing, China). The optical density (OD) value was measured at 520 nm (for tissue iron) and 405 nm (for GSH). Iron and GSH concentrations were calculated following the manufacturer’s instructions.

Malondialdehyde test
Tissue from rats at 7 dpi was collected as described above. Samples were lysed in 9-fold (volume/weight) of cell lysis buffer (R0130, Beyotime Biotechnology) followed by centrifugation at 12,000 × g for 10 minutes. All steps were performed at 4°C or on ice. Supernatant was processed using the malondialdehyde (MDA) assay kit (S0131S, Beyotime Biotechnology). The OD value was measured at 532 nm and the MDA concentration was determined by comparing the OD of the standards.

ROS assay
A ROS essay kit (WLA131, WanleiBio, Shenyang, China) was used to detect the ROS content of each group at 7 dpi. After removal of spinal dura mater and nerve roots, spinal cords were homogenized by pipetting in pancreatic for 2 minutes. Homogenate was filtered with a 200 mesh and the cellular suspension was incubated with 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM) at 37°C for 25 minutes. Samples were imaged with a microscope (Olympus, Tokyo, Japan) and quantification of ROS level was analyzed by software Imagej v1.53c (National Institutes of Health, Bethesda, MD, USA; Schneider et al., 2012).

Quantitative reverse transcription polymerase chain reaction
Spinal cords from rats at 7 dpi were lysed in Trizol (1 mL for 100 mg tissue). Total RNA was extracted with chloroform, precipitated with isopropyl alcohol and rinsed with 75% ethanol. Purity and concentration of RNA were determined following the OD 260/280 nm method (reference value: 1.8–2.0 for purity; 300–400 ng/μL for concentration). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the Evo M-MLV RT Master Mix (AG11706, Accurate Biotechnology, Shanghai, China) following the manufacturer’s instructions; the 10 μL reaction contained 2 μL Evo M-MLV RT Master Mix and 500 ng total RNA in Rnase free water. The reaction conditions were 73°C for 15 minutes, 95°C for 5 seconds and 4°C as the final step. The primers were designed using the BLAST module of NCBI (Table 1). qRT-PCR was performed using a 2 μL reaction containing 10 μL 2× SYBR Green Pro Taq HS Premix (AG111701, Accurate Biotechnology), 70 μL complementary DNA, 0.4 μL forward primer, 0.4 μL reverse primer and RNase free water on a LightCycler 96 (Roche, Alameda, CA, USA). The cycling conditions were as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds, concluding with 95°C for 10 seconds, 65°C for 60 seconds and 97°C for 1 second. All samples were run in duplicate, and the data were quantitated by the 2^-ΔΔCt method (Livak and Schmittgen, 2001).

Table 1 | Primer sequence used in quantitative reverse transcription polymerase chain reaction

| Gene | Primer sequence (5’–3’) | Product size (bp) |
|------|--------------------------|------------------|
| xCT  | Forward: GGTC CAG AGC ATG CAG AGA CAA | 189 |
|      | Reverse: ACC AAT TCC TTT AGC CCA TCA | |
| Gpx4 | Forward: AGT CCG AGG GGC CCC GGA | 170 |
|      | Reverse: CAT GGC GGT ATT CAC ACA AG | |
| β-actin | Forward: CAC CAT GTA CCC AGG CAT TG | 173 |
|      | Reverse: CTC GCT TGA TCA ACC ACA TC | |

Gpx4: Glutathione peroxidase 4; xCT: glutamate/cystine reverse transporter system xCT

Cell culture and viability assay
PC12 were an alternative cell line for primary neurons (Greene and Tischler, 1976), were purchased from the Cell Resource Center of Shanghai Institute for Biological Science (Shanghai, China), Cat# TCR8, Identifier code: CSTR:19375.09.3101RATTCR8). PC12 cells are widely used as a model cell of neurons to investigate neuronal damage resulting from SCI (Lin et al., 2016; He et al., 2017; Shen et al., 2017). Cells were cultured at 37°C and 5% CO2 in Roswell Park Memorial Institute 1640 Medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, San Diego, CA, USA). The half maximal inhibitory concentration (IC50) of RSL3 to PC12 cells was used for en bloc staining for 2 hours, and then samples were serially dehydrated with 50%, 70%, 90% and 100% alcohol and embedded in epoxy resin for making blocks. Silver sections were cut with an ultramicrotome (EM UC7, Leica; thickness 70–90 nm) and stained with lead citrate and uranyl acetate. The sections were observed and imaged with a transmission electron microscope (Talos L120C, Thermo Scientific, Waltham, MA, USA).

Tissue iron and reduced GSH assay
Rats at 7dpi were anesthetized and perfused with pre-cooling sterile saline for removal of erythrocytes in spinal cords. The injured tissue was moved to a sterile Petri dish. Spinal dura mater and nerve roots were carefully peeled off. The tissues were homogenized in 9-fold (volume/weight) of normal saline. After centrifugation at 600 g for 10 minutes, the supernatant was collected and assayed with a tissue iron assay kit (AO39-2-1) and reduced-GSH assay kit (AO60-2-1). Nanjing Jianghe Biotechnology Co., Ltd., Nanjing, China). The optical density (OD) value was measured at 520 nm (for tissue iron) and 405 nm (for GSH). Iron and GSH concentrations were calculated following the manufacturer’s instructions.
Results

EPO exhibits a neuroprotective effect in SCI rat

To determine whether EPO exerts a neuroprotective effect in SCI rats, we evaluated the recovery of hindlimb motor function in the four experimental groups using the BBB scale (scored at 1, 3, 7, 14 dpi) and footprint analysis (at 14 dpi). At 1 and 3 dpi, there was no significant difference in BBB score in the four groups relative to that in the vehicle group (vehicle vs. EPO: P = 0.608; high dose vs. vehicle: P = 0.534; Figure 1B). However, from day 7, the BBB scores in the EPO groups were significantly higher than those in the vehicle group (low dose vs. vehicle: P = 0.022; high dose vs. vehicle: P < 0.001). Footprint analysis showed that the stride distance of the EPO groups was remarkably improved, and that the bilateral strides of the EPO groups were more symmetrical than those of the vehicle group (low dose vs. vehicle: P = 0.005 for left and P < 0.001 for right; high dose vs. vehicle: P < 0.001 for both sides; Figure 1C and D). The higher BBB score and longer stride distance in the high dose group relative to those in the low dose group indicated that high dose EPO had a better neuroprotective effect on SCI than the low dose (P < 0.001).

EPO promotes regeneration of SCI

Histology and neuroimaging were used to evaluate the regeneration of injured spinal cord at 14 dpi. HE staining was performed to observe the tissue damage and repair, and Nissl staining was used to evaluate the loss of spinal neurons. In T2 fluorescence intensity in the injured group relative to the sham group (Figure 1E). MRI scans were performed to evaluate the lesion areas within the spinal cord. Fat-suppressed T2-weighted sagittal MRI showed that the hypointense area of the injured cord was significantly reduced in the EPO treated group compared with the vehicle group (Figure 1F). Moreover, the vehicle group had a much higher level of tissue iron concentration relative to the sham group, the iron concentration decreased with the treatment of EPO (P < 0.001; Figure 2B).

EPO diminishes the accumulation of ROS and lipid peroxidation in the injured spinal cord

In ferroptosis, iron overload generates highly reactive hydroxy radicals through the Fenton reaction, which results in the accumulation of ROS and lipid peroxidation (Jing et al., 2021). MDA is a natural product of lipid oxidation in living organisms, reflecting the level of lipid peroxidation. We next evaluated ROS and MDA contents respectively. Fluorescence analysis revealed that the ROS fluorescence intensity in the vehicle group was significantly increased compared to that in the EPO groups (Figure 2A). Moreover, the vehicle group had a much higher level of tissue iron concentration relative to the sham group, the iron concentration decreased with the treatment of EPO (P < 0.001; Figure 2B).

EPO attenuates SCI-induced expression of ferroptosis markers

Accumulating evidence demonstrates that ferroptosis is involved in neuronal death following SCI (Zhang et al., 2020; Cheng et al., 2021; Feng et al., 2021; Gao et al., 2021). We next examined the expression of the ferroptosis-related proteins Tfr, Fpn, Acs4 and 4-Hne in injured spinal cords at 14 dpi (Feng et al., 2020; Cui et al., 2021; Rui et al., 2021) using western blot assay. All markers were increased in the vehicle group relative to those in the sham group (Figure 3A–H). We found that 4-Hne was significantly downregulated in both low and high dose EPO groups relative to that in the vehicle group (low dose vs. vehicle: P = 0.001; high dose vs. vehicle: P = 0.001), while Tfr, Acs4, Fpn and Fth were decreased in the high dose group (Tfr: P = 0.016; Acs4: P = 0.005; Fpn: F < 0.001; Fth: F = 0.003).

EPO upregulates the expression of xCT and Gpx4 and rescues depletion of GSH in SCI

Gpx4 and xCT have a synergistic relationship in the antioxidant system, and their reduction can trigger ferroptosis (Kremer et al., 2021; Lu et al., 2022). No animals or data points were excluded from the analysis. The statistical analysis and visualization were performed using GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA). All analyzed data were evaluated by analysis of variance followed by Tukey’s multiple comparisons test or one-way analysis of variance followed by Dunnett’s multiple comparisons test. P < 0.05 indicated statistical significance.

Mitochondrial membrane potential test

Mitochondrial membrane potential (MMP) of PC12 cells was detected using an enhanced MAM assay kit with JC-1 (C20035, Beyotime Biotechnology). Cells were cultured with JC-1 working solution (JC-1 100×, JC-1 buffer = 1:200) at 37 °C for 5 min. An inverted fluorescence microscope (Axio Observer 3, Oberkochen, Germany) was used for observation, and flow cytometry (FCM, Bd Celesta, San Jose, CA, USA) was used for quantitative analysis.

Immunofluorescence staining

Spinal cord tissues were fixed, embedded in paraffin, cut into 4 μm sections and slices were blocked and permeabilized in immune staining solution (PBS) for 20 min at room temperature. Sections were then incubated with fluorescence secondary antibody (37 °C for 1 hour): fluorescein isothiocyanate (FITC; rabbit, 1:100, ZSGB-BIO, Beijing, China, Cat# ZF-0311, RRID: AB_2571576), tetramethylrhodamine isothiocyanate (TRITC; rabbit, 1:100, ZSGB-BIO, Cat# ZF-0312, RRID: AB_2716306), tetramethylrhodamine isothiocyanate (TRITC; rabbit, 1:100, ZSGB-BIO, Cat# ZF-0311, RRID: AB_2571576), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; rabbit, 1:500, Affinity, Cat# AF7021, RRID: AB_2839421). The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit (1:10,000, Affinity, Cat# S0001, RRID: AB_722490) or anti-mouse secondary antibodies (1:10,000, Affinity, Cat# S0002, RRID: AB_2839430) at 25°C for 1.5 hours. Protein bands were visualized using an enhanced chemiluminescence system (ECL Western Blotting Detection Reagents, Shanghai Chemical Reagent Co., Ltd., China). Quantitation of western blot was performed by software ImageJ v1.53c. Gapdh was used as internal reference.

Mitochrondial functions were determined using an enhanced MMP assay kit with JC-1 (C20035, Beyotime Biotechnology). Cells were cultured with JC-1 working solution (JC-1 100×, JC-1 buffer = 1:200) at 37 °C for 5 min. An inverted fluorescence microscope (Axio Observer 3, Oberkochen, Germany) was used for observation, and flow cytometry (FCM, Bd Celesta, San Jose, CA, USA) was used for quantitative analysis.
EPO promotes hindlimb function recovery and tissue repair after SCI. High dose EPO attenuates iron overload, ROS accumulation and MDA content in the injured spinal cord.

Neuroprotection of EPO in SCI is largely abolished by the ferroptosis activator RSL3

RSL3 is a ferroptosis activator targeting Gpx4 (Shin et al., 2018), and Fer-1 is a ferroptosis inhibitor (Liu et al., 2020b). We evaluated neuronal function recovery and ferroptosis level in SCI rats treated with EPO, EPO + RSL3 and Fer-1. We further determined the expression of ferroptosis-related proteins in the EPO and Fer-1 groups were significantly lower than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups. The expressions of xCT and Gpx4 in the EPO and Fer-1 groups were significantly higher than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups.

We next analyzed the expressions of ferroptosis-related proteins. The expressions of Tfr, Fpn and 4-Hne in the EPO and Fer-1 groups were significantly lower than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups. The expressions of xCT and Gpx4 in the EPO and Fer-1 groups were significantly higher than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups.

Figure 1 | EPO promotes hindlimb function recovery and tissue repair after SCI. (A) A schematic of the animal experiments. (B) BBB scores of the indicated groups. (C) Footprints of the hindlimb at 14 dpi. (D) The boxplot of stride distance for footprint analysis. (E) HE (top row) and Nissl staining (bottom row) of injured tissue at 14 dpi. (F) MRI of each group at 14 dpi. The arrows indicate the epicenter of lesions. All data are expressed as the mean ± SD (n = 5 in each group). ***P < 0.001, ###P < 0.001, vs. vehicle group; ###P = 0.001, vs. low dose group (two-way analysis of variance followed by Tukey’s multiple comparisons test). BBB: Basso, Beattie and Bresnahan locomotor rating scale; dpi: day(s) post injury; GSH: glutathione; HE: hematoxylin eosin; IF: immunofluorescence; MDA: malondialdehyde; L: left; MRI: magnetic resonance imaging; qRT-PCR: quantitative reverse transcription polymerase chain reaction; R: right; ROS: reactive oxygen species; SCI: spinal cord injury; TEM: transmission electron microscope; WB: western blot.

Figure 2 | EPO attenuates iron overload, ROS accumulation and MDA content in the injured spinal cord. (A) Perls’ Prussian blue staining of injured tissue at 7 dpi. Iron-positive cells were stained by blue. Scale bars: top, 50 μm; bottom, 25 μm. (B) Iron contents of injured tissue at 7 dpi. (C) A schematic of the animal experiments. (D) BBB scores of the indicated groups. (E) Low dose of EPO vs. vehicle: P = 0.005 for Tfr and Fpn, P = 0.02 for 4-Hne; EPO + RSL3: P = 0.005 for Tfr, P = 0.002 for Fpn, P = 0.01 for 4-Hne; Fer-1 vs. vehicle: P < 0.001 for Tfr and Fpn, P = 0.005 for 4-Hne; Fer-1 vs. EPO vs. RSL3: P = 0.004 for Tfr, P = 0.014 for Fpn, P = 0.013 for 4-Hne; Figure 4B–E). The expressions of xCT and Gpx4 in the EPO and Fer-1 groups were significantly higher than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups. The expressions of xCT and Gpx4 in the EPO and Fer-1 groups were significantly higher than those in the vehicle group, while the expression in the EPO + RSL3 group was increased relative to that in the EPO and Fer-1 groups.
To further confirm the putative role of EPO on SCI-induced ferroptosis, we used TEM to observe the mitochondrial morphology. Ferroptosis-like morphological changes, characterized by the reduction or vanishing of mitochondria cristae and outer mitochondrial membrane rupture (Liu et al., 2020a), were observed in the vehicle group and the EPO + RSL3 group (Figure 4H). The mitochondria in the EPO and Fer-1 groups were morphologically closer to those of the sham group.

Our results indicated that EPO attenuated ferroptosis within the local tissue after SCI, but whether EPO exerted its effects via inhibiting neuronal ferroptosis was unclear. Therefore, double-label immunofluorescence of spinal cord sections was used to determine the expression of xCT, Gpx4 and 4-Hne in spinal neurons. There were more cells positive for NeuN (a neuronal marker) in the EPO and Fer-1 groups than the vehicle and EPO + RSL3 groups (Figure 5). Furthermore, the fluorescence intensities of xCT and Gpx4 were higher in the EPO and Fer-1 groups relative to that in the vehicle group (xCT: *P* < 0.001 for both EPO and Fer-1; Gpx4: *P* = 0.004 for EPO, *P* = 0.011 for Fer-1), while 4-Hne showed the opposite trend. There were no significant differences in the staining for xCT, Gpx4 and 4-Hne in the EPO + RSL3 and vehicle groups (xCT: *P* = 0.998; Gpx4: *P* = 0.273; 4-Hne: *P* = 0.974). Collectively, our results showed that the therapeutic effect of EPO on SCI rats was related to ferroptosis inhibition which was mediated by upregulating xCT and Gpx4.
EPO rescues RSL3-induced ferroptosis in PC12 cells

To more closely examine the effects of EPO on SCI-induced ferroptosis in rats, we performed in vitro experiments in PC12 cells. Cell viability assays of RSL3 treatment in PC12 cells were performed, and the IC50 of RSL3 at 24 hours was 10.21 μM (Figure 6A). We then examined the inhibitory effect of EPO at different concentrations (5, 10, 20, 40 IU/mL) against RSL3-induced ferroptosis. EPO at 40 IU/mL showed the most significant suppression on Tfr and 4-Hne (P = 0.006 and P = 0.003 respectively, relative to that at 0 IU/mL; Figure 6B–D). Therefore, RSL3 at 10 μM and EPO at 40 IU/mL were selected to establish the RSL3 + EPO group for the subsequent assays.

Loss of MMP is a feature of early ferroptosis (Chiu et al., 2009). MMP assay with JC-1 showed that the MMP of the RSL3 group decreased, and after treatment with EPO, the MMP was closer to that of the control group (Figure 6E). Moreover, FCM results showed that the number of JC-1 monomer-positive cells in the RSL3 + EPO group was significantly less than that of the RSL3 group (P < 0.001; Figure 6F and G). These results suggested that EPO may improve mitochondrial state in RSL3-treated PC12 cells.

As shown in Figure 7A–D, there was a significant reduction of 4-Hne and a significant increase of xCT and Gpx4 in the RSL3 + EPO group relative to the levels in the RSL3 group (4-Hne: P = 0.003; xCT: P = 0.048; Gpx4: P = 0.019). Immunofluorescence staining revealed stronger fluorescence intensity of xCT and Gpx4 and weaker 4-Hne in the RSL3 + EPO group relative to the RSL3 group (Figure 7E–J). These results suggested that EPO protected PC12 cells from RSL3-induced ferroptosis via upregulating xCT and Gpx4.

Discussion

In the present study, we used a rat SCI model and PC12 cell model to investigate the mechanism of the neuroprotective effects of EPO and potential involvement of ferroptosis. We used two different doses of EPO to treat SCI rats, and both doses of EPO improved the hindlimb function recovery and spinal cord regeneration. Several characteristics of ferroptosis, including iron overload, ROS accumulation, lipid peroxidation, GSH depletion and ferroptosis biomarkers (Dixon et al., 2012; Zheng et al., 2020), were rescued by EPO. High dose of EPO exhibited a stronger inhibitory effect on SCI-induced ferroptosis relative to low EPO dose. RSL3 is a Gpx4-targeting ferroptosis inducer (Shin et al., 2018), and Fer-1 is a potent and selective ferroptosis inhibitor (Liu et al., 2020a). The efficacy of EPO was similar to that of Fer-1, and the effects of EPO disappeared when co-treated with RSL3. The RSL3-induced lipid peroxidation and exhausation of xCT and Gpx4 in PC12 cells was reversed by EPO.
BBB scale and footprint analysis are two classical methods to evaluate the neurological function recovery of SCI rats (Basso et al., 1995; Wen et al., 2015). Our results demonstrated that EPO promoted the BBB scores and stride distance after SCI even with a low dose. The improvement in hindlimb motor function was consistent with the findings of histology and MRI scan. Tissue necrosis and neuron loss were relieved by both doses of EPO. The MRI scan was performed at 14 dpi; therefore, we speculate that the high signal on sagittal T2-weighted within spinal cord did not represent the edema but the ischemia/reperfusion injury, which is an important pathogenic factor contributing to neurological deterioration (Liao et al., 2020). This area of high signal was significantly reduced by EPO treatment.

Iron overload is a characteristic and driving factor of SCI-induced ferroptosis (Dixon et al., 2012; Jing et al., 2021). Erythrocyte lysis following SCI causes the sharp increase of ferric ion (Fe³⁺) locally. Fe³⁺ is transported into cells mediated by Tfr and degraded to the highly reactive Fe²⁺ by the action of xCT, a transmembrane protein of prostate 3, which induces ferroptosis through the Fenton reaction (Chen et al., 2020). When intracellular iron accumulates, Fth and Fpn, which function in iron storage and export, respectively, are upregulated (Rui et al., 2021). Therefore, Tfr, Fpn and Fth are regarded as the indicators of ferroptosis. We found that Tfr, Fpn and Fth were significantly decreased in response to EPO high dose and iron overload was alleviated with EPO. Therefore, high dose of EPO might stabilize the cellular iron metabolism in SCI-induced ferroptosis.

Ferroptosis is also characterized by ROS accumulation and lipid peroxidation (Zheng et al., 2020). Fenton reaction catalyzed by iron generates a large amount of ROS, which leads to cell death (Hassania et al., 2019). Increased polyunsaturated fatty acid is a cause of lipid peroxidation (Hong et al., 2017), and Acsl4 is one of the key enzymes involved in polyunsaturated fatty acid formation (Doll et al., 2017). Dehydrogenation of polyunsaturated fatty acids forms phospholipid hydroperoxides, which is decomposed to 4-Hne and MDA (Hassania et al., 2019). As the end products of lipid peroxidation, 4-Hne and MDA reflect the degree of ferroptosis. In our study, we found weaker fluorescence of ROS, downregulated expression of 4-Hne and lower level of MDA in response to EPO. These findings suggested a reduction of ferroptosis in EPO groups.

Gpx4 plays an essential role in ferroptotic regulation (Xie et al., 2016). Polyunsaturated fatty acid forms phospholipid hydroperoxide that is converted by Gpx4 to phosphatidylcholine (PL-alcohol), which does not exert lipid radical activity. Therefore, Gpx4 can prevent lipid peroxidation and suppress ferroptosis (Proneth and Conrad, 2019). GSH is a cofactor for Gpx4 and important for maintaining Gpx4 activity (Chen et al., 2020). Cysteine is an indispensable substrate for GSH synthesis. Cellular cysteine is transported into cells via xCT, which comprises two subunits: solute carrier family 3 member 2 and the solute carrier family 7 member 11 (Bordini et al., 2020). We observed reduction of GSH, xCT and Gpx4 in the vehicle group after SCI, which was consistent with previous reports (Zhang et al., 2019, 2020). However, we found the markedly augmented GSH and upregulated mRNA and protein of xCT and Gpx4 in the two EPO groups.

While both doses of EPO exhibited neuroprotective and anti-ferroptotic effects, there were no significant differences between the groups in ferroptosis biomarker expression. Tfr, Acsl4, Fpn and Fth expressions decreased significantly with high EPO dose not with low dose. Results from the behavior test, ROS, iron content and MDA assays in the high dose group were superior to those in the low dose group. Therefore, low EPO dose exhibited a stronger effect on activating xCT and Gpx4, but it was not enough for maintaining iron homeostasis and promoting functional recovery. Considering the stronger effects in the high dose group, we used the high EPO dose for the second animal experiments.

The neuroprotective and anti-ferroptotic effects of EPO were largely offset by RSL3 in vivo. The decreased BBB scores in EPO + RSL3 group could partly explain the results of double-labeled fluorescence staining: EPO promoted the neuronal xCT and Gpx4 to alleviate ferroptosis while RSL3 reproduced neuronal ferroptosis. The biomarkers related to ferroptosis (Tfr, Fpn and 4-Hne) were upregulated and the enhanced effects of EPO on xCT and Gpx4 were abolished in the EPO + RSL3 group. Additionally, the ferroptosis inhibitor Fer-1 and EPO exerted the similar effects on SCI rats. In vitro, we validated the promotion of EPO on xCT and Gpx4 using EPO to rescue RSL3-induced ferroptosis in PC12 cells.

Mitochondrial dysfunction is intrinsically linked to ferroptosis. Shrunken mitochondrial and disappeared cristae are morphological characteristics of SCI-induced ferroptosis (Zhang et al., 2019; Ge et al., 2021). MMP is an effective biomarker to assess the function of the mitochondria (Galley et al., 2017) and decreased MMP is one of the early symbols involved in ferroptosis (Chiu et al., 2009; Wu et al., 2018; Ge et al., 2021). We found that EPO and Fer-1 alleviated the mitochondria damage after SCI as shown in TEM results. Fluorescence and flow cytometry indicated that EPO contributes to the recovery of decreased MMP in ferroptosis. This study has several limitations. First, the sample size was relatively small, and an increased sample size would be considered to improve the accuracy of our study. Second, primary neuronal cells should be used to better understand the neuroprotective effect of EPO.

In conclusion, we examined the effects of EPO on preventing SCI-induced ferroptosis in a rat SCI model and determined the facilitatory effects of EPO on xCT and Gpx4 in vivo and in vitro. These findings provide new insights into the neuroprotective effect of EPO in SCI, and the anti-ferroptotic effect of EPO makes it a potential therapeutic strategy for clinical improvement of SCI.

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