Sodium selenite promotes neurological function recovery after spinal cord injury by inhibiting ferroptosis

Abstract

Ferroptosis is a recently discovered form of iron-dependent cell death, which occurs during the pathological process of various central nervous system diseases or injuries, including secondary spinal cord injury. Selenium has been shown to promote neurological function recovery after cerebral hemorrhage by inhibiting ferroptosis. However, whether selenium can promote neurological function recovery after spinal cord injury as well as the underlying mechanism remain poorly understood. In this study, we injected sodium selenite (3 μL, 2.5 μM) into the injury site of a rat model of T10 vertebral contusion injury 10 minutes after spinal cord injury modeling. We found that sodium selenite treatment greatly decreased iron concentration and levels of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal. Furthermore, sodium selenite increased the protein and mRNA expression of specificity protein 1 and glutathione peroxidase 4, promoted the survival of neurons and oligodendrocytes, inhibited the proliferation of astrocytes, and promoted the recovery of locomotive function of rats with spinal cord injury. These findings suggest that sodium selenite can improve the locomotive function of rats with spinal cord injury possibly through the inhibition of ferroptosis via the specificity protein 1/glutathione peroxidase 4 pathway.

Key Words: ferroptosis; glutathione peroxidase 4; glutathione; iron; lipid peroxidation; neural regeneration; secondary injury; sodium selenite; specificity protein 1; spinal cord injury

Introduction

Spinal cord injury (SCI) represents a severe form of trauma to the central nervous system and poses a serious threat to health in humans (No authors listed, 2016). The pathophysiology of SCI can be assigned to primary and secondary phases. Irreversible primary injury from mechanical forces leads to the compression or contusion of the spinal cord, while secondary injury occurs because of the development and perpetuation of the primary injury (Mourelo Fariña et al., 2017). The causes of secondary injury include ischemic edema, oxygen free radical production, calcium overload, inflammatory reactions, excitatory amino acid release, and necrosis or apoptosis initiation, which ultimately lead to loss of neural function. Thus, to treat primary SCI, it may be necessary to reduce or inhibit associated secondary injury.

In recent years, a new mode of cell death known as ferroptosis (Dixon et al., 2012) has been found to be involved in the pathological process of secondary injury associated with SCI (Shi et al., 2021). Ferroptosis is induced by intracellular iron overload and accumulation of lipid-derived reactive oxygen species (ROS). Compared with apoptosis, necrosis, and autophagy, ferroptosis has its own unique characteristics with regard to morphology, biochemistry, and genetic regulation (Dixon et al., 2012). The most important hallmarks of ferroptosis are the loss of capacity to repair lipid peroxides by glutathione peroxidase 4 (GPX4), oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids, and availability of redox-active iron (Dixon and Stockwell, 2019).

Currently, ferroptosis is considered to be triggered by iron overload followed by excessive generation of noxious lipid peroxides, which affect membrane permeability as well as integrity and eventually leads to cell damage (Hassannia et al., 2019). During these processes, lysophosphatidylcholine acyltransferase 3 (LPCAT3) and acyl-coenzyme A synthetase long-chain family member 4 (ACSL4) enable dense clustering of high-sensitivity PUFAs in cell membranes. Lipoxigenases, especially 15-lipoxygenase (Yang et al., 2016), oxidize PUFAs and generate intermediate products of lipid peroxidation known as lipid hydroperoxides, which include phosphatidylethanolamine-linked arachidonic acid or arachidonic acid. These intermediate products accumulate within the plasma membrane and release ferroptosis signals (Wenzel et al., 2017). In the presence of Fe²⁺ within the cells, lipid hydroperoxides generate lipid-derived ROS, which cause irreversible cellular damage and death.

Previous studies have suggested that multiple pathways are involved in ferroptosis. In the GPX4 pathway, GPX4 is involved in conversion of toxic lipid-derived ROS to lipid hydroperoxides, which is then converted to lipid-derived ROS by the lipid peroxidation products malondialdehyde and 4-hydroxynonenal. These intermediate products accumulate within the plasma membrane and release ferroptosis signals (Wenzel et al., 2017). In the presence of Fe²⁺ within the cells, lipid hydroperoxides generate lipid-derived ROS, which cause irreversible cellular damage and death.
Recent research has confirmed that intracerebroventricular injection of sodium selenite (SS) significantly improved neurological recovery in a mouse model of ICH (Alim et al., 2019). We hypothesized that SS would also effectively improve neurological recovery after SCI. In this study, we evaluated the neural protective effects of SS in a rat model of SCI, investigated the changes in factors essential for ferroptosis, and determined whether GPx4 and ferroptosis were targets by which SS exerted its effects on neural repair.

Materials and MethodsExperimental animals
The urethra of female rats is short and wide, which is convenient for spinal cord injection. Therefore, this study was conducted with 10-week-old, female Wistar rats (220 ± 20 g). The rats were purchased from Sibefu Biotechnology Co., Ltd. (Beijing, China; license No. SCXK [Jing] 2019-0010). The rats were maintained in a temperature- and humidity-controlled room with a 12-hour dark/light cycle and free access to water and food. The Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China) approved this study (approval No. AEEI-2019-041) on March 25, 2019. The study was conducted in accordance with the Code of Ethics for the Purpose of Control and Supervision on Experiments on Animals (CPCEA) guidelines.

SS preparation
SS (Na2SeO3; product number S5261) was purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). The SS powder was dissolved in normal saline at a stock concentration of 10 μM and diluted to working concentrations of 5 and 2.5 μM. The stock solutions of SS were frozen at −20°C. In accordance with the product description, the working aliquots were protected from light using a dark container and were used within 2 weeks.

For molecular and biochemical analysis, 40 mg/kg sodium pentobarbital solution (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally to anesthetize the rats followed by transcardial perfusion with phosphate-buffered solution (PBS). Then, a 1.5-cm long spinal cord segment, including the injury epicenter (center of congestion and swelling, including the injury epicenter), was resected and frozen in liquid nitrogen as described previously (Qin et al., 2018). For pathological, immunofluorescence, and immunohistochemical analysis, rats were anesthetized with intraperitoneal pentobarbital sodium injection, transcardiac perfusion was performed with saline followed by 4% paraformaldehyde, and the spinal cord samples were resected and stored in 4% paraformaldehyde at 4°C.

Rats were sacrificed on days 1, 3, and 7 post-surgery, and spinal cord samples were harvested immediately and fresh frozen in liquid nitrogen followed by storage at −80°C for analysis. At 8 weeks post-injury, rats were sacrificed and spinal cord samples were collected for preparation of paraffin sections. Then, 5-μm-thick sections were dewaxed with xylene and anhydrous alcohol and stained.

Basso, Beattie and Bresnahan locomotor rating scale scores
Functional recoveries of hind limbs were assessed by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). The BBB score was assigned from 0 to 21, where 0 indicates no movement and 21 indicates normal locomotion. The BBB score was assessed immediately (time 0) and then once per week for 8 weeks post-surgery, and these assessments were conducted by two independent observers who were blinded to the experimental groups.

Gait analysis
Gait analysis was performed 1 day before contusion injury and at week 8 postoperatively (the endpoint of the experiment). Quantification of gait data was performed using the DigiGait Imaging System (Mouse Specifics, Boston, MA, USA). Briefly, DigiGait™ automatically identified and analyzed gait data from the video files. The accuracy rate of the rat was on the average better than 50 metrics. In this study, the treadmill speed was 10 cm/second, and all dynamic gait indices were calculated using the DigiGait analysis software. The indices that were analyzed included paw area, hind limb shared stance time, circle, and paw symmetry. The paw area refers to the maximal paw area that was in contact with the treadmill during the stance phase of the step cycle. The hind limb shortening and stride time refer to the time that both hind limbs touched the belt with the belt. The stride duration refers to the time during which the paw remained in contact with the belt. The stride duration refers to the time required for one limb to complete a single stride. Stride frequency refers to the number of complete stride cycles performed by one paw per second. Stride length refers to the distance between the initial contacts of the same paw in one complete stride cycle of the hind limbs. The formula by which gait symmetry was calculated is as follows: (right forelimb step frequency + left forelimb step frequency) / 2 – (right forelimb step frequency – left forelimb step frequency). A gait symmetry value closer to 1 represented improved coordination of forelimbs and hind limbs.
Transmission electron microscopy

Spinal cord tissue samples from the three groups were collected at 24 hours after injury, and the samples were immersed in a solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate, fixed in 4% paraformaldehyde in 1% sodium cacodylate, stained with 1% uranyl acetate, dehydrated in alcohol and acetone. After embedding in epoxy resin, the samples were polymerized for 48 hours at 60°C. Ultrathin sections (60–80 nm) were cut using an ultramicrotome and stained with 1% uranyl acetate and lead citrate. Transmission electron microscopy (H7700; Hitachi, Tokyo, Japan) was used for imaging.

Evaluation of iron levels

Iron concentrations in spinal cord specimens were determined on days 1, 3, and 7 after injury using an iron assay kit (A003-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer’s instructions. Briefly, the samples were vortexed for 1 minute for which the tube was sealed with preservative film, and a small hole was drilled with a needle. The tubes were placed in a boiling water bath for 40 minutes, cooled, and centrifuged for 10 minutes at 986–1127 × g. The ODs of the resulting supernatants were measured at 532 nm with a spectrophotometer (Leidi Life Technology Co., Ltd., Shenzhen, China).

Determination of MDA concentrations

MDA levels in spinal cord specimens were evaluated on days 1, 3, and 7 post-injury using an MDA assay kit (A003-1, Nanjing Jiancheng Bioengineering Institute) following the manufacturer’s instructions. Briefly, the samples were vortexed for 1 minute for which the tube was sealed with preservative film, and a small hole was drilled with a needle. The tubes were placed in a boiling water bath for 40 minutes, cooled, and centrifuged for 10 minutes at 986–1127 × g. The ODs of the resulting supernatants were measured at 532 nm with a spectrophotometer (Leidi Life Technology Co., Ltd., Shenzhen, China).

Determination of glutathione concentrations

Glutathione (GSH) concentrations in the T10 spinal cord specimens were measured using the rubrization method in immunoradiometric precipitation assay buffer (Servicebio, Wuhan, China) supplemented with protease inhibitors using a tissue homogenizer (Servicebio). The protein levels were evaluated with a bicinchoninic acid kit (Servicebio). Following denaturation, equal protein amounts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels). After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), which were blocked for 1 hour at room temperature using Tris-buffered saline-Tween 20 containing 5% skim milk. Primary antibodies were diluted, added to the membranes, and incubated at 4°C overnight. The primary antibodies used in this study are shown in Table 1. The membranes were washed three times with Tris-buffered saline-Tween 20 followed by incubation with a secondary antibody (horseradish peroxidase [HRP]-conjugated goat anti-mouse IgG; 1:5000; Servicebio, Cat# GB23303, RRID: AB_2904020 or HRP-conjugated goat anti-rabbit IgG; 1:5000; Servicebio, Cat# GB23303, RRID: AB_2904018 or Cy3-conjugated goat anti-rabbit IgG; 1:500; Servicebio, Cat# GB23303, RRID: AB_2861435) for 5 minutes in the dark at room temperature. After three washes with PBS, the nuclei were stained with 2% uranium acetate saturated alcohol solution and lead citrate. The membranes were washed three times with Tris-buffered saline-Tween 20 containing 5% skim milk. Primary antibodies were diluted, added to the membranes, and incubated at 4°C overnight. The primary antibodies used in this study are shown in Table 1. The membranes were washed three times with Tris-buffered saline-Tween 20 followed by incubation with a secondary antibody (horseradish peroxidase [HRP]-conjugated goat anti-mouse IgG; 1:5000; Servicebio, Cat# GB23303, RRID: AB_2904020 or HRP-conjugated goat anti-rabbit IgG; 1:5000; Servicebio, Cat# GB23303, RRID: AB_2904018 or Cy3-conjugated goat anti-rabbit IgG; 1:500; Servicebio, Cat# GB23303, RRID: AB_2861435) for 5 minutes in the dark at room temperature. After three washes with PBS, the nuclei were stained with 4',6-diamidino-2-phenyldine for 10 minutes. The sections were incubated with a anti-fluorescence quenching medium. The numbers of neuronal nuclear antigen (NeuN)-positive cells and glial fibrillary acidic protein (GFAP)-positive cells were imaged by fluorescence microscopy (Eclipse C1; Nikon, Tokyo, Japan).

Immunohistochemistry

Antigen retrieval within spinal cord sections was conducted using ethylenediaminetetraacetic acid buffer (pH 9.0). The sections were incubated with 3% hydrogen peroxide at room temperature for 25 minutes in the dark to block endogenous peroxidase activity. Then, bovine serum albumin was added by a following 30 minute incubation. Diluted primary antibodies (Table 1) were added dropwise, and the sections were incubated at 4°C overnight. The sections were washed with PBS, the corresponding secondary antibodies (described above) were added to the encircled sections, and the sections were incubated for 5 minutes in the dark at room temperature. Color development was performed with 3,3'-diaminobenzidine, and the nuclei were stained with hematoxylin for 3 minutes. After dehydration, the sections were mounted with neutral gum and imaged by microscopy (XSP-C204; Chongqing Institute of Neurology Co., Chongqing, China). After hematoxylin staining, nuclei appeared blue, while positive expression via 3,3'-diaminobenzidine appeared brownish yellow. The number of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)-positive cells in the SCI group was calculated.

**Table 1** | Primaries antibodies used in this study

| Antibody | Host | Dilution | Catalog No. | RRID | Supplier | Application |
|----------|------|----------|-------------|-------|----------|-------------|
| GPX4 | Rabbit | 1:2000 | ab125066 | AB_10973901 | Abcam | WB |
| SP1 | Mouse | 1:1000 | sc-420 | AB_262871 | Santa | WB, IF |
| 4-HNE | Rabbit | 1:3000 | ab46545 | AB_722490 | Abcam | WB |
| NeuN | Mouse | 1:200 | GB1331B-1 | AB_2904014 | Servicebio | IF |
| GFAP | Rabbit | 1:800 | GB11096 | AB_2904015 | Servicebio | IF |
| CNPase | Rabbit | 1:50 | 13427-I-AP | AB_280247 | Proteintech | IHC |
| β-actin | Mouse | 1:1000 | GB12001 | AB_2904016 | Servicebio | WB |

**Table 2** | Primers for real-time quantitative polymerase chain reaction

| Gene | Primer sequence (5′–3′) | Accession number | Product size (bp) |
|------|----------------------|------------------|------------------|
| ACSL4 | Forward: TAC AGG ATT CCT CCA AGT AGC Reverse: CAG CGG TAG GTA AAG GAC | NM_053623.1 | 236 |
| LPCAT3 | Forward: CCT ACT TCT AGG CAG CCT TCT TGG T | NM_00101289.1 | 224 |
| GPX4 | Forward: GCC GAG TGT GGT TTA CGA ATC Reverse: AGG CAG CAG TTT CTC GTA | NM_000398493.3 | 257 |
| CRP3 | Forward: GCA CAT TAA CCT CAG TGC A | NM_012655.2 | 142 |
| GAPDH | Forward: CTG GAG AAT CTC GGC AAT T Reverse: GGT GGA AGA ATG GGA GTT | NM_017008.4 | 138 |

ACS4: Acyl-coenzyme A synthetase long-chain family member 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPX4: glutathione peroxidase 4; LPCAT3: lysophosphatidylcholine acyltransferase 3; SP1: specificity protein 1.

**Immunofluorescent staining**

Antigen retrieval within spinal cord sections was conducted using a citric acid buffer solution (pH 6). The sections were circled using a marking pen, and autofluorescence quencher solutions (G1221, Servicebio), which effectively reduce spontaneous tissue fluorescence and improve the signal-to-noise ratio of immunofluorescence detection. Sections were washed three times with PBS, incubated for 5 minutes in the dark at 4°C. After washing with PBS, the sections were incubated with bovine serum albumin (Servicebio) for 30 minutes. Diluted primary antibodies (Table 1) were added dropwise, and the sections were incubated at 4°C overnight. After washing for 10 minutes, the sections were incubated with the corresponding secondary antibodies (Alexa Fluor® 488-conjugated goat anti-mouse IgG; 1:400; Servicebio, Cat# GB25301, RRID: AB_2904018 or Cy3-conjugated goat anti-rabbit IgG; 1:300; Servicebio, Cat# GB23303, RRID: AB_2861435) for 5 minutes in the dark at room temperature. After three washes with PBS, the nuclei were stained with 4',6-diamidino-2-phenyldine for 10 minutes. The sections were incubated with a anti-fluorescence quenching medium. The numbers of neuronal nuclear antigen (NeuN)-positive cells and glial fibrillary acidic protein (GFAP)-positive cells were imaged by fluorescence microscopy (Eclipse C1; Nikon, Tokyo, Japan).
Histological analysis
The spinal cord samples were embedded in paraffin and cut into 5-μm-thick sections. The sections were stained with hematoxylin-eosin reagents (ServaBio). The results were observed using an optical microscope (Eclipse E100; Nikon) and included the number of cells, arrangement regularity, and cavity sizes.

Statistical analysis
GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA; www.graphpad.com) and SPSS (IBM SPSS Statistics for Windows (2015), version 23.0; IBM Corp., Armonk, NY, USA) software programs were used for analyses. Between-group comparisons were performed using the nonparametric Scheirer-Ray-Hare test with Dunn’s post hoc test. Data are shown as median and interquartile range, and *P < 0.05 denoted significance.

Results
SS improves hind limb locomotion recovery in rats with SCI
Figure 1 shows the BBB scores of rats in the three groups as a function of time post-SCI. Immediately following SCI, rats in the SCI and SS groups exhibited severe hind limb paralysis. Hind limb motor function began to recover at 1 week after SCI and showed a gradual increase during the 8-week experimental period. The BBB group were markedly lower than those in the SCI group at each assessment point from 2–8 weeks after SCI. These results suggested that SS treatment promoted functional recovery after SCI.

Figure 1 | SS treatment improves recovery of hind limb motor function in SCI rats. (A) Design of the study. The baseline gait analysis data were obtained 1 day before the experiment. The rats were allocated into three groups (Sham, SCI, and SS groups). In the SS group, 3 μL of SS (2.5 μM) was injected into the spinal cord 10 minutes after SCI. Transmission electron microscopy was used to assess the injured spinal cord 24 hours after contusion. Western blot analysis, real-time quantitative polymerase chain reaction, immunofluorescent staining, and other methods were used to detect changes in factors related to ferroptosis on days 1, 3, and 7 after SCI. Hematoxylin-eosin, immunofluorescent, and immunohistochemistry staining were performed 8 weeks after injury. The BBB scores were evaluated each week until the 8th week, and gait analysis was performed again at the 8th week. (B) Alterations in BBB scores for rat hind limbs. The BBB scores for hind limbs in the SS group were significantly higher than those in the SCI group beginning at week 2 after injury. The data are shown as medians and interquartile ranges (n = 6). *P < 0.05; **P < 0.01. (C) SCI: Spinal cord injury; SS: sodium selenite; TEM: transmission electron microscope.

Baseline gait imaging was performed 1 day before SCI and repeated 8 weeks after SCI at a predetermined speed of 10 cm/s. Typical gait images of rats from the three groups at 8 weeks post-SCI are shown in Figure 2A. At 8 weeks after establishment of the model, the SCI group demonstrated a significant decrease in hind limb paw area (P = 0.004), and SS treatment ameliorated this loss (P = 0.006). Gait analysis revealed a significant decrease in hind limb shared stance time in the SCI group (P = 0.004) relative to that in the Sham group. In addition, the hind limb shared stance time in the Sham group was significantly greater than that in the SCI group (P = 0.004; Figure 2C). The hind limb stance duration and stride duration of the rats in the SCI group were markedly lower than those in the Sham group (P = 0.008, P = 0.01), and SS treatment did not result in significant improvements (P = 0.109, P = 0.106; Figure 2D and E). A distinct increase in hind limb stride frequency in the SCI group (P = 0.013) was observed relative to that in the Sham group at 8 weeks post-injury; however, SS treatment did not alleviate this change (P = 0.055; Figure 2F). Stride length was significantly reduced in the SCI at 8 weeks after injury (P = 0.01), but the effect of SS treatment did not reach statistical significance (P = 0.092, Figure 2G). Compared with that in the Sham group, SCI significantly increased gait symmetry after 8 weeks (P = 0.006), and SS administration significantly alleviated this alteration (P = 0.02; Figure 2H).

SS treatment affects factors essential for ferroptosis in spinal cord tissues after SCI
Iron concentrations, Acsl4 and Lpcat3 mRNA expression, MDA levels, and HNE expression were detected at 1, 3, and 7 days after SCI. Relative to that in the Sham group, the SCI group showed markedly higher iron levels (P < 0.05), and iron concentrations in the SS group were statistically lower than that in the SCI group at 7 days after injury (P = 0.037; Figure 4A). These data indicated that iron overload may have been a critical factor that triggered ferroptosis after SCI, which was alleviated by SS treatment.

Acsl4 and Lpcat3 are involved in the biosynthetic pathway of PUFAs and can induce ferroptosis in vivo (Doll et al., 2017; Kagan et al., 2017). RT-qPCR demonstrated that Acsl4 and Lpcat3 mRNA expression was upregulated in spinal cord tissues of the SCI group compared with expression in the Sham group on days 1 and 3 post-injury (P < 0.05); however, differences between the two groups 7 days after injury were insignificant (P > 0.05). Moreover, SS treatment did not significantly affect Acsl4 and Lpcat3 mRNA expression (P > 0.05; Figure 4B and C). Considering that Acsl4 and Lpcat3 are associated with lipid peroxidation (Doll et al., 2017; Kagan et al., 2017), we examined the lipid peroxidation products MDA and 4-hydroxynonenal (4-HNE) in the SCI group compared with those from the Sham group (P < 0.05), and SS treatment partially alleviated this change (P < 0.05; Figure 4D–F). Taken together, the above results demonstrated that lipid peroxidation catalyzed by Acsl4 and Lpcat3 occurred in spinal cord tissues after SCI, and SS treatment decreased the iron concentration and levels of hemolysis.
lipid peroxidation. However, ACSL4, and LPCAT3 do not appear to be targets of SS.

As a crucial molecule in the signaling pathway that regulates ferroptosis, GPX4 catalyzes the reduction of lipid peroxides. GPX4 inactivation is one of the key features of ferroptosis, and this inactivation occurs either by decreasing GSH levels or by directly targeting GPX4 (Dixon and Stockwell, 2019). Relative to that in the Sham group, the SCI group exhibited significantly lower GSH expression at all time points post-injury (P < 0.05), and GPX4 expression was significantly decreased on days 1 and 3 after injury (P < 0.05). Additionally, SS treatment led to statistically significant improvement in GSH and GPX4 expression (P < 0.05; Figure 4E–F).

Together, major factors in the ferroptosis pathway, including iron, ACSL4, LPCAT3, MDA, 4-HNE, and the GSH/GPX4 axis, were linked to secondary injury associated with SCI. Furthermore, SS treatment suppressed ferroptosis by inhibiting iron accumulation, activating GPX4, and curbing lipid peroxidation.

**Possible mechanisms by which SS improves neural function recoveries after SCI**

We determined the biomechanism by which SS promoted GPX4 expression and inhibited ferroptosis. Sp1 and Gpx4 mRNA and protein expression and GPX enzymatic activity was analyzed on days 1, 3, and 7 after SCI. Relative to expression in the Sham group, the SCI group showed markedly lower GPX4 protein and Gpx4 mRNA expression (Figures 4H, I and 5A). GPX activity was also reduced by injury (Figure 5B). SS treatment led to marked increases in these indicators.

Western blot, RT-qPCR, and immunofluorescent staining revealed that SP1 protein and mRNA expression was decreased after SCI, most noticeably within 3 days, and SS treatment improved the expression of these molecules substantially at all three time points post-injury (Figure 5C–G). Taken together, SS may upregulate GPX4 expression by increasing the expression of the transcription factor Sp1, thereby enhancing GPX4 activity, suppressing ferroptosis, and promoting neurological recovery after SCI.
SS promotes spinal cord repair after SCI

Hematoxylin-eosin staining of spinal cord tissues at 8 weeks post-injury is shown in Figure 6. The structures of spinal cord tissues in the Sham group were basically normal, whereas the SCI group displayed spinal cord tissue breakdown, scar connections, structural disorder, and obvious cavity formation. Comparatively, the cavities within the spinal cord tissues of the SS group were significantly reduced, and tissue arrangement demonstrated greater regularity. Thus, we inferred that SS promoted repair of the injured spinal cord tissue and, subsequently, improved hind limb motor function.

Figure 6 | Effects of SS treatment on spinal tissue repair after SCI. Hematoxylin-eosin staining was performed on cross sections of the injury epicenter 8 weeks after SCI. The nuclei are blue, and the cytoplasm is red. Representative images showed obvious cavities and tissue disorder (arrows) in the SCI group. In contrast to the SCI group, the cavities were significantly reduced, and the tissue arrangement was more regular in the SS group. Scale bars: 500 µm (middle panels), 100 µm (right panels). SCI: Spinal cord injury; SS: sodium selenite.

SS increases the number of NeuN+ and CNP cells but decreases the number of GFAP+ cells after SCI

We performed immunofluorescent staining and immunohistochemical analysis to identify potential target cells that may benefit from SS treatment in rats with SCI. Immunofluorescence staining revealed that the numbers of NeuN+ cells in the SCI group were significantly decreased relative to those in the Sham group at 8 weeks post-injury, whereas the numbers of NeuN+ cells in the SS group were increased compared with those in the SCI group. In comparison, the numbers of GFAP+ cells in the SCI group were substantially higher relative to those in the Sham group at 8 weeks after injury, whereas the SS group showed a slight increase (Figure 7A–C). The differences between the SCI and SS groups were statistically significant for both proteins.

Immunohistochemical results demonstrated that the numbers of CNP+ cells in the SCI group were markedly decreased compared with those in the Sham group at 8 weeks after injury (Figure 7D). SS treatment caused a substantial increase in the numbers of CNP+ cells compared with that in the SCI group. Because CNP is a specific marker of oligodendrocytes and myelinated fibers (Müller and Seifert, 1982), we concluded that SS treatment protected oligodendrocytes during the pathology of SCI.

Discussion

Cell death is a major contributor to secondary injury associated with SCI, and the mechanisms of cell death include apoptosis, necrosis, and ferroptosis (Shi et al., 2021). Inhibition of ferroptosis represents a theoretically feasible treatment for SCI. Previous studies on iron chelators and specific lipid peroxidation scavengers have demonstrated that ferroptosis inhibitor significantly improved the motor function of SCI rats (Yao et al., 2019; Zhou et al., 2020). Our results indicated that ferroptosis occurred in spinal cord lesions after contusion. Furthermore, several factors that initiate and regulate ferroptosis exhibited SCI-induced alterations, while SS intervention effectively ameliorated these changes. The histomorphological and staining results observed in this study also support similar conclusions. The present study showed that SS exerted neuroprotective effects after SCI. More importantly, for the first time, we demonstrated that the neuroprotective effects of SS may be achieved by inhibiting ferroptosis mediated by GPX4.

Data from BBB scores and gait analysis showed that SS administration after SCI enhanced hind limb locomotion recovery, which agrees with results from previous studies (Yeo et al., 2008; Chen et al., 2013). However, it should be noted that this study focused on improvement of neurological function from the perspective of inhibiting ferroptosis rather than apoptosis. Using electron microscopic analysis, we observed that SCI caused numerous typical manifestations of ferroptosis, while SS intervention reduced these changes. Neither the SS group nor the SCI group exhibited typical manifestations of apoptosis. A previous study demonstrated that cell apoptosis occurred 1–2 days after injury (Nottingham and Springer, 2003), which may explain these results. SS administration significantly reduced empty cavities and resulted in more uniform tissue structure. In addition, SS administration increased the percentage of NeuN+ and CNP+ cells but decreased the number of GFAP+ cells in spinal cord tissues. Thus, we suggest that SS promoted repair after

Figure 7 | Effects of SS on the numbers of NeuN+, GFAP+, and CNP+ cells after SCI. (A) The effect of SS on NeuN and GFAP immunofluorescence at 8 weeks after injury. Immunofluorescent images of NeuN+ and GFAP+ cells in the epicenter of injury were captured by fluorescence microscopy. Representative fluorescence micrographs of NeuN (green, Alexa Fluor 488), GFAP (red, Cy3), and nuclei (blue, DAPI) at the epicenter of injury. NeuN+ cells in the SCI group were decreased compared with that in the Sham group, whereas NeuN+ cells in the SS-treated group were increased relative to that in the SCI group. GFAP+ cells in the SCI group were increased compared with that in the Sham group, whereas the GFAP+ cells in the SS-treated group were decreased compared with that in the SCI group. Scale bar: 100 µm. (B, C) Quantification of NeuN+ and GFAP+ cells. The data are shown as medians and interquartile ranges (n = 3). *P < 0.01 (Scheirer-Ray-Hare test followed by Dunn’s post hoc test). (D) The effect of SS on CNP immunohistochemical staining at 8 weeks after injury. Images of the transected sections of the epicenter of injury were captured by microscopy. Representative micrographs of CNP staining (yellowish-brown) and nuclear staining (blue) at the epicenter of injury. CNP+ cells in the SCI group were markedly decreased compared with that in the Sham group at 8 weeks after injury, whereas SS treatment caused a marked increase in the number of CNP+ cells (arrows). Scale bar: 100 µm. (E) The possible mechanism by which SS promotes the recovery of neurological function in rats with SCI through GPX4. 4-HNE: 4-Hydroxynonenal; AA: arachidonic acid; AA-CoA: arachidonic acid-coenzyme A; ACSI4: acyl-coenzyme A synthetase long-chain family member 4; AdA: adrenic acid; AdA-CoA: adrenic acid-coenzyme A; ACSI4: acyl-coenzyme A synthetase long-chain family member 4; AdA: adrenic acid; AdA-CoA: adrenic acid-coenzyme A; CNPase: 2′,3′-cyclic nucleotide-3′-phosphodiesterase; DAPi: 4′,6-diamidino-2-phenylindole; GFAP: glial fibrillary acidic protein; GPX4: glutathione peroxidase 4; GSH: glutathione; LPCAT3: lysophosphatidylcholine acyltransferase 3; MDA: malondialdehyde; NeuN: neuronal nuclear antigen; PE-AAA: phosphatidylethanolamine-arachidonic acid; PE-AdA: phosphatidylethanolamine-adrenic acid; ROS: reactive oxygen species; SCI: spinal cord injury; SP1: specificity protein 1; SS: sodium selenite.

SCI by preserving neurons and oligodendrocytes, which increased nerve fiber regeneration and remyelination, and by inhibiting reactive astrogliosis and glial scar formation.

To further investigate the mechanism by which SS protects neurons in rats with SCI, we conducted a study at the molecular level. The results revealed that the iron concentrations in injured spinal cord tissues were markedly elevated in the SCI group, and SS administration partially alleviated this increase. Iron chelators have previously been shown to inhibit ferroptosis by dramatically decreasing the iron load within spinal cord lesions (Yao et al., 2019). Therefore, the mechanistic action of SS may differ from that of iron chelators. ACSI4 and LPCAT3 are key enzymes responsible for the synthesis and reconstruction of PUFAs that are incorporated into cell membrane phospholipids. Overexpression of these enzymes can increase the levels of substrates available for lipid peroxidation, which subsequently triggers ferroptosis (Doll et al., 2017; Kagan et al., 2017). RT-qPCR studies revealed
Several signaling pathways regulate ferroptosis through GPX4, including the glutamate cysteine reverse transporter system (system XC−/GSH/GPX4 axis). System XC− is a heterodimer composed of the light chain subunit SLC7A11 (XCT) and heavy chain subunit SLC3A2 linked through disulphide bonds and mediates the 1:1 exchange of intracellular glutamate and extracellular cystine (Dixon et al., 2019). The XCT subunit contains a selenocysteine insertion sequence which is the raw material for the synthesis of GSH. Erastin inhibits system XC− resulting in the inhibition of GSH synthesis (Sato et al., 2018). The lack of GSH and GPX4 suppresses the removal of lipid peroxides, which then results in membrane damage and ferroptosis induction (Sellek et al., 2008; Hassaniy et al., 2019).

In the ICH model, Alim et al. (2019) found that human brain cells autonomously regulated cell damage caused by hemorrhage through Se intake. Further studies showed that selenium upregulated the expression of GPX4, inhibited ferroptosis, and protected neurons through activation of the transcription cofactor SP1. The 3′ untranslated region of GPX4 mRNA contains a selenocysteine insertion sequence element (Touat-Hamici et al., 2014). During translation, the UGA codon is usually read as the termination codon, while in the presence of the selenocysteine insertion sequence, the UGA codon encodes a selenocysteine (U46). This particular form of translation requires a unique protein system to guide the insertion of selenocysteine into GPX4 and other selenoproteins. Therefore, the expression of GPX4 is regulated by the availability of selenium (Min et al., 2018). Both SCN and SS are central nervous system components involved in the production of GPX4, and selenium functioned via a similar mechanism in SCI. Our results suggest that SS may increase the levels of the transcription factor SP1 and upregulate the expression of GPX4, thus inhibiting ferroptosis (Figure 7E).

Selenium has been previously used in the treatment of SCI; however, its role in protecting neural function has been studied from the perspective of inhibiting neuron apoptosis (Yes et al., 2008) and upregulating the expression of cardiovascular neurological factor and its receptor (Chen et al., 2015). In this study, the role of selenium in improving neural function after SCI was examined for the first time from the perspective of inhibiting ferroptosis. It should be noted that selenium-mediated regulation of GPX4 and protection of neurons are complex multifactorial processes rather than simple linear mechanisms. Therefore, the cross effects of various mechanisms, including different pathways of cell regeneration, microenvironment changes, and oxidative stress regulation, may have influenced our experimental results.

Because of the short experimental time, there were limitations in the present study. For the selenium/SP1/GPX4 axis, we only demonstrated a positive correlation in their expression levels without determining direct binding relationships and exact mechanisms. Therefore, the conclusions require verification in future studies. For ACSL4 and LPCAT3, we only measured mRNA expression without protein expression and activity levels of them. Because transcription, translation, and activity are not always linked, the conclusion that “ACSL4 and LPCAT3 do not seem to be targets of SS” is not reliable enough. Thus, experiments evaluating protein expression and activity levels of ACSL4 and LPCAT3 should be performed in future researches. In terms of gait analysis, more gait indices and time points should be considered to obtain greater detail and more representative results. Only rat experiments were conducted in the present study to demonstrate the effectiveness of selenium. Subsequent studies should combine in vivo and in vitro experiments that include different types of SCI models. The sample size for each group was limited, and follow-up studies should include expanded sample sizes to provide more power to the results.

In the present study, SS was administered by local spinal cord injection, which may increase the risk of infection. Moreover, SS is reported to have a narrow safe concentration range for spinal cord injection, which may increase the likelihood of toxicity issues. Therefore, future studies will be required to explore safer and more effective methods for the administration of SS.

In summary, SCI increased the concentration of iron and lipid peroxidation levels and decreased the expression of GPX4, thus triggering the onset of ferroptosis. SS administration after SCI administration is located to downregulate expression of ACSL4 and LPCAT3 and is closely related to the elimination of lipid peroxidation.

Ferroptosis has become an important research topic in recent years, and the molecular mechanism of this process has been continuously studied. Among various ferroptosis regulatory molecules, GPX4 is considered to be the core regulator of ferroptosis. In contrast, the inactivation or inhibition of GPX4 leads to a breakdown in oxidation balance, and lipid peroxidation are able to destroy the cell membrane structure and trigger ferroptosis (Dixon and Stockwell, 2019). To elucidate the relationship between SS treatment and GPX4 changes in SCI, we conducted a study. Following SCI, levels of components in the GPX4 pathway decreased sharply, which agrees with previous literature (Yao et al., 2019). Additionally, SS increased GPX4 expression and activity and ultimately improved neurological functions by suppressing ferroptosis.

In summary, SCI increased the concentration of iron and lipid peroxidation. In SCI, the expression of cardiovascular neurological factor and its receptor (Chen et al., 2019) and the selenocysteine insertion sequence in the ACSL4 gene, which is the raw material for the synthesis of GSH. Erastin inhibits system XC− resulting in the inhibition of GSH synthesis (Sato et al., 2018). The lack of GSH and GPX4 suppresses the removal of lipid peroxides, which then results in membrane damage and ferroptosis induction (Sellek et al., 2008; Hassaniy et al., 2019).

In conclusion, SS increased the concentration of iron and lipid peroxidation levels and decreased the expression of GPX4, thus triggering the onset of ferroptosis. SS administration after SCI administration is closely related to the elimination of lipid peroxidation and promoting neurological recovery.
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