Original Article

Astragaloside IV ameliorates spinal cord injury through controlling ferroptosis in H_2O_2-damaged PC12 cells *in vitro*

Yifei Zhou[1,2], Lin Li[1], Chenghuang Mao[1], Dongsheng Zhou[1,3]

[1]Department of Traumatology and Orthopedics, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China; [2]Department of Traumatology and Orthopedics, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, China; [3]Department of Traumatology and Orthopedics, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China

Contributions: (I) Conception and design: D Zhou, Y Zhou; (II) Administrative support: D Zhou; (III) Provision of study materials or patients: L Li, C Mao; (IV) Collection and assembly of data: Y Zhou; (V) Data analysis and interpretation: Y Zhou; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Dongsheng Zhou; Yifei Zhou. Department of Traumatology and Orthopedics, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, No. 44, Wenhua West Road, Lixia District, Jinan 250021, China. Email: sdgkxh@aliyun.com; yifeizhou@wmu.edu.cn.

Background: Spinal cord injury (SCI) is associated with significant paralysis and high fatality. Recent research has revealed that ferroptosis participates in the pathogenesis of SCI. Astragaloside IV (AS-IV), the main active ingredient of the plant *Astragalus membranaceus*, has been reported to promote motor function recovery in rats with SCI. This study explored the effects of AS-IV in H_2O_2-treated PC12 pheochromocytoma cells.

Methods: The optimal concentration and duration of AS-IV treatment in PC12 cells was assessed using the cell counting kit 8 (CCK-8) assay. Subsequently, the SCI cell model was established in PC12 cells using H_2O_2. The effects of AS-IV, FIN56, and transcription factor EB (TFEB) small interfering (si)RNA on cell viability and apoptosis in the SCI model were determined using the CCK-8 assay and flow cytometry, respectively. Caspase-3 and lactate dehydrogenase (LDH) levels were measured by colorimetric assay and enzyme-linked immunosorbent assay (ELISA), respectively. Cellular reactive oxygen species (ROS) were detected by flow cytometry combined with dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. The cellular ultrastructure was analyzed by transmission electron microscopy (TEM). The ferroptosis pathway-related proteins were confirmed using Western blot analysis. TFEB expression was confirmed by Western blot and immunofluorescence.

Results: The optimal concentration and duration of AS-IV treatment in PC12 cells was determined to be 1.0 μM and 48 h, respectively. AS-IV markedly accelerated proliferation, suppressed apoptosis, and reduced ROS and LDH accumulation. Furthermore, AS-IV enhanced TFEB expression in H_2O_2-damaged PC12 cells. The effects of AS-IV on SCI were inhibited by si-TFEB, and this inhibition was further reinforced by the addition of FIN56.

Conclusions: The results of this investigation using the SCI cell model suggested that AS-IV alleviated SCI by promoting TFEB expression and subsequently mediating ferroptosis. This may represent a potential clinical treatment for SCI.

Keywords: Astragaloside IV (AS-IV); spinal cord injury (SCI); ferroptosis; transcription factor EB (TFEB)

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Introduction

Spinal cord injury (SCI) is a temporary or permanent damage to the spinal cord structure and function (1). SCI often causes a number of neurological disorders, including limb pain, numbness, weakness, paralysis, and urinary and fecal disorders, all of which may be life-threatening (2). The etiology of SCI can be classified into traumatic and non-traumatic factors, with the former being the main cause of SCI (3,4). Traumatic SCI refers to acute injury of the spinal cord caused by external physical impacts, such as sports-related injuries, motor vehicle injuries, violence, or falls (5). Non-traumatic SCI refers to injury to the spinal cord in the course of an acute or chronic disease, including infections, degenerative diseases, or tumors (6). Recent epidemiological data revealed that there are about 23 new SCI cases per million people worldwide annually (7). SCI has a high incidence and can cause severe disability, especially in young adults (8). Currently, the treatment modalities of SCI include surgery and nonsurgical therapies, the latter of which mainly includes drug therapy, vascular booster therapy, stem cell therapy, hypothermia therapy, growth factor therapy, and electrical stimulation of the spinal cord (9,10). However, management of SCI patients is associated with high costs and poor curative rates. Therefore, the prevention and treatment of SCI is a challenging global medical and social problem.

Astragalus membranaceus (Ast) is a Traditional Chinese medicine that is used for tonifying middle-Jiao and Qi, inducing diuresis to alleviate edema, promoting the elimination of toxins, and the production of new tissues (11). Astragaloside IV (AS-IV), the main active ingredients of Ast, has a powerful antioxidant effect by removing free radicals and reducing lipid peroxidation (12). To date, AS-IV has been demonstrated to have multiple pharmacological effects, including anti-diabetes, anti-hypertension, myocardial protection, anti-heart failure, anti-inflammation, and anti-infarction (12,13). Furthermore, both *in vitro* and *in vivo* studies have shown that AS-IV can distinctly reduce the levels of reactive oxygen species (ROS) to alleviate oxidative damage (14,15). There is an overproduction of ROS after SCI and this facilitates the cascade of secondary injuries (16). ROS clearance can prevent or relieve the secondary injury after SCI (17). Therefore, we hypothesized that AS-IV may have certain protective effects against SCI. However, the specific functions and mechanisms of AS-IV in SCI remain to be fully elucidated.

In the past decade, study has shown that apoptosis, autophagy, necrosis and pyroptosis can regulate the occurrence and development of SCI (18). Lately, as a new term, ferroptosis has been paid more and more attention in SCI (19). Ferroptosis is an iron-dependent form of non-apoptotic cell death driven by excessive iron accumulation and lipid peroxidation (20). Ferroptotic cells are characterized by mitochondrial abnormalities such as swelling, increased membrane density, crest loss, and rupture of the outer membrane (21). Recently, some studies have shown that ferrostatin-1, an inhibitor of ferroptosis, improved recovery of spinal cord function after injury (22,23). Therefore, ferroptosis may be a potential therapeutic target for SCI. In fact, report suggests that inhibiting iron death is helpful to alleviate spinal cord injury (24). For example, Ge *et al.* find that Zinc inhibits ferroptosis and alleviates spinal cord injury through Nrf2/GPX4 pathway (25). In addition, AS-IV has been shown to reduce ROS production and improve oxidative stress (26). However, whether AS-IV can alleviate SCI through ferroptosis and the regulatory mechanisms involved remains to be elucidated. Therefore, this current study established a SCI model *in vitro* by H_2O_2 pretreatment of PC12 pheochromocytoma cells, to observe the function of AS-IV in SCI. The underlying mechanisms of AS-IV in H_2O_2-damaged PC12 cells were analyzed. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5196/rc).

Methods

Cell culture

The PC12 pheochromocytoma cell line was obtained from the National Infrastructure of Cell Line Resource (Beijing, China). Cells were grown in RPMI1640 medium (Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco, Cat. No. 10270) at 37 ℃ in a 5% CO_2 incubator. To induce a nerve cell phenotype, cells were treated with 0.5 μg/mL nerve growth factor (NGF) for 5 days.

Cell treatment

PC12 cells were incubated with 0, 0.01, 0.1, 1, 10, or 100 μM AS-IV (HPLC ≥98.0%, Cat No. JZ16042403) for 0, 24, 48, 72, and 96 h. Negative control (NC) small interfering (si)RNA and si-transcription factor EB (TFEB) were obtained from GenePharma (Shanghai, China). The H_2O_2-treated PC12 cell model was established using

300 μmol/L H_2O_2. The H_2O_2-damaged PC12 cells were treated with 0.1 μM AS-IV and 5 μM FIN56 (AbMole, USA), which is a specific inducer of ferroptosis. Cells were transfected with NC siRNA and si-TFEB using Lipofectamine 3000 (Life Technologies).

Cell counting kit 8 (CCK-8) assay

After specific treatment, PC12 cells were harvested and seeded into 96-well plates at 5,000 cells/well and maintained at 37 ℃ for 24 h. Cells were then incubated with 10 μL CCK-8 for 1 h at 37 ℃. The optical density (OD) value at 450 nm was determined using a microplate reader.

Caspase-3 activity assay

Caspase-3 activity was determined using the Caspase-3 Assay Kit (ab39401, Abcam). Briefly, proteins were harvested from PC12 cells and the concentration was adjusted. Caspase-3 activity was then determined in accordance with the manufacturer's instructions.

Measurement of intracellular reactive oxygen species

To determine the levels of ROS, PC12 cells were harvested and exposed to 10 μM DCFDA (abcam, USA) in serum-free medium at 37 ℃ for 45 min in the dark. Cells were then washed with phosphate buffered saline (PBS) and the intensity of fluorescent DCF at 485/535 nm (ex/em) was measured using a fluorescence microscope. Additionally, flow cytometry (FACScan, USA) was performed to determine the levels of ROS in PC12 cells. The data was analyzed using CELL Quest 3.0 software.

Enzyme-linked immunosorbent assay (ELISA)

PC12 cells were harvested and seed into 6-well plates at 1×10^4 cells/well. After treatment with H_2O_2, AS-IV, FIN56, or si-TFEB, the cell supernatant was harvested and the lactate dehydrogenase (LDH) concentrations were determined using the LDH ELISA Kit (Roche, Germany) as per the manufacturer's protocols.

Transmission electron microscopy (TEM)

Treated PC12 cells were harvested and incubated with 2% glutaraldehyde (Cat. no. 354400) overnight at 4 ℃. The cells were then fixed with 1% osmic acid at 4 ℃ for 2 h, followed by washing and staining with uranyl acetate (Electron Microscopy Sciences, Cat. No. 22,400). Cells were then dehydrated with 70%, 80%, 90%, and 100% acetone, sequentially. After pre-embedding, the cells were embedded with Epon812 epoxy resin (Hede Biotechnology Co., Ltd., Beijing, China) and stained with toluidine blue (Solarbio). Following sectioning, the results were observed under a TEM (JEOL, Tokyo, JEM-1011).

Western blot

To extract the total protein, treated PC12 cells were lysed using RIPA buffer (Cell Signaling Technology) supplemented with protease inhibitor (Sigma-Aldrich, Cat. no. P8340). The nuclear and cytoplasmic proteins were acquired by applying Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). After protein quantification, the samples were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking by skin milk, the proteins were immersed in an appropriate dilution of the primary antibodies, and the corresponding secondary antibody. Individual protein bands were visualized using the electrochemiluminescence (ECL) kit (Amersham Biosciences). The primary antibodies used were SLC7A11 (Abcam, ab175186), GPX4 (Abcam, ab125066), TFEB (Abcam, ab270614), GAPDH (Cell Signaling Technology), and horse radish peroxidase (HRP)-labeled secondary antibody (Cell Signaling Technology, Bioke).

Immunofluorescence assay

The treated PC12 cells were seeded in a 6-well plate with coverslips and incubated at 37 ℃ for 6 h. After washing with pre-cooled PBS, PC12 cells were fixed with 40 g/L paraformaldehyde for 20 min, and treated with Triton X-100 (10 mL/L) for 15 min. After blocking with 100 mL/L goat serum for 30 min, the cells were incubated with anti-TFEB (1:200, Abcam, ab270614) at 4 ℃ overnight, followed by the CY5-labeled secondary antibody (1:200, Abcam) at 37 ℃ for 1 h. Cells were then stained with 100 μL DAPI (100 g/L) for 5 min in the dark. TFEB expression was visualized and photographed under a fluorescence microscope.

Statistical analysis

The data herein are presented as mean ± standard deviation

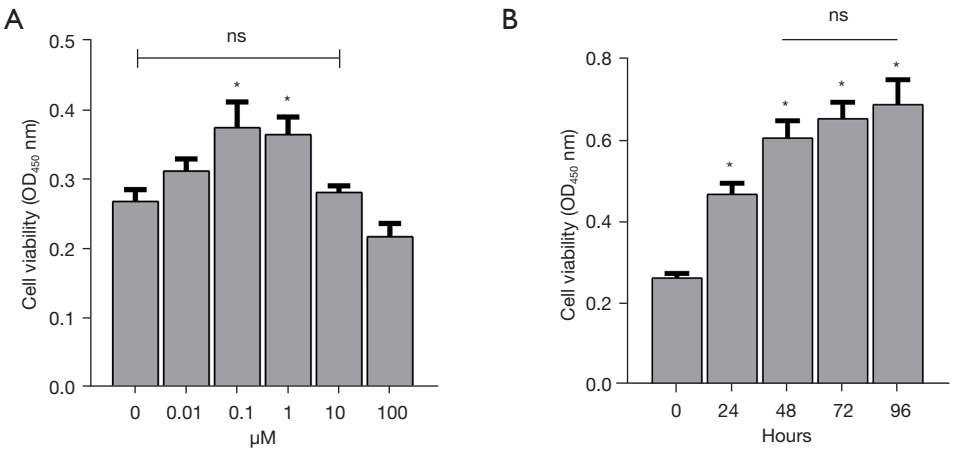

Figure 1 The concentration and during of AS-IV exposure were confirmed in the cultured PC12 cells. (A) PC12 cells were treated with 0, 0.01, 0.1, 1, 10, and 100 μM AS-IV for 24 h. The CCK-8 assay was conducted to examine the proliferative activity of PC12 cells. (B) PC12 cells were treated with 0.1 μM AS-IV for different durations (0, 24, 48, 72, and 96 h) and the proliferative activity the cells was determined using the CCK-8 assay. *P<0.05; ns, no statistical significance; AS-IV, astragaloside IV; CCK-8, cell counting kit 8.

(SD) based on 3 replicates, and analyzed using one-way analysis of variance (ANOVA). All statistical analyses were performed using the SPSS20.0 software. A P value <0.05 was considered statistically significant.

Results

The impacts of AS-IV on the viability of the cultured PC12 cells

To investigate the influence of AS-IV on PC12 cells, cell viability and proliferation were examined using the CCK-8 assay after incubation with 0, 0.01, 0.1, 1.0, 10, and 100 μM AS-IV for 24–96 h. As displayed in *Figure 1A*, 0.1 μM and 1 μM AS-IV significantly increased the viability of PC12 cells compared to untreated cells. Indeed, the enhanced cell viability was more pronounced with 0.1 μM AS-IV than with 1.0 μM AS-IV. AS-IV (0.1 μM) also significantly enhanced the proliferative activity of PC12 cells after treatment for 24–96 h, especially after 48, 72, and 96 h (*Figure 1B*). After a comprehensive analysis, a concentration of 0.1 μM AS-IV was adopted for the all subsequent experiments with an incubation time of 48 h.

AS-IV protects PC12 cells against injury induced by H_2O_2 and enhances TFEB expression

Accumulating evidence suggests that an excess of ROS plays a crucial role in secondary injury cascades of SCI (27). To further investigate the protective role of AS-IV on SCI, 300 μmol/L H_2O_2 was used to induce the OS (oxidative stress) of PC12 cells, and the H_2O_2-treated PC12 cells were then incubated with 0.1 μM AS-IV. Cell viability was markedly reduced in the H_2O_2-treated cells compared to untreated control cells. However, incubation with AS-IV significantly increased the viability of H_2O_2-treated PC12 cells (*Figure 2A*). In addition, H_2O_2 markedly induced the apoptosis of PC12 cells, and this could be reversed by treatment with AS-IV (*Figure 2B*). Furthermore, H_2O_2 significantly elevated the levels of caspase 3 and LDH in PC12 cells, and this was ameliorated by treatment with AS-IV (*Figure 2C,2D*). H_2O_2-treated cells showed suppressed expression of TFEB compared to control cells, and its expression was reversed after AS-IV administration (*Figure 2E,2F*). These results demonstrated that AS-IV effectively alleviated the H_2O_2-induced effects on PC12 cells.

AS-IV suppressed ferroptosis in H_2O_2-treated PC12 cells

The role of ferroptosis in the protective effects of AS-IV on H_2O_2-treated PC12 cells was examined. Flow cytometry showed that AS-IV reduced the ROS production induced in H_2O_2-treated PC12 cells (*Figure 3A,3B*). TEM demonstrated that the mitochondria in the H_2O_2 group were small and atrophied, and the mitochondrial ridges were decreased or even absent. Interestingly, AS-IV reversed the mitochondrial morphology observed in H_2O_2-

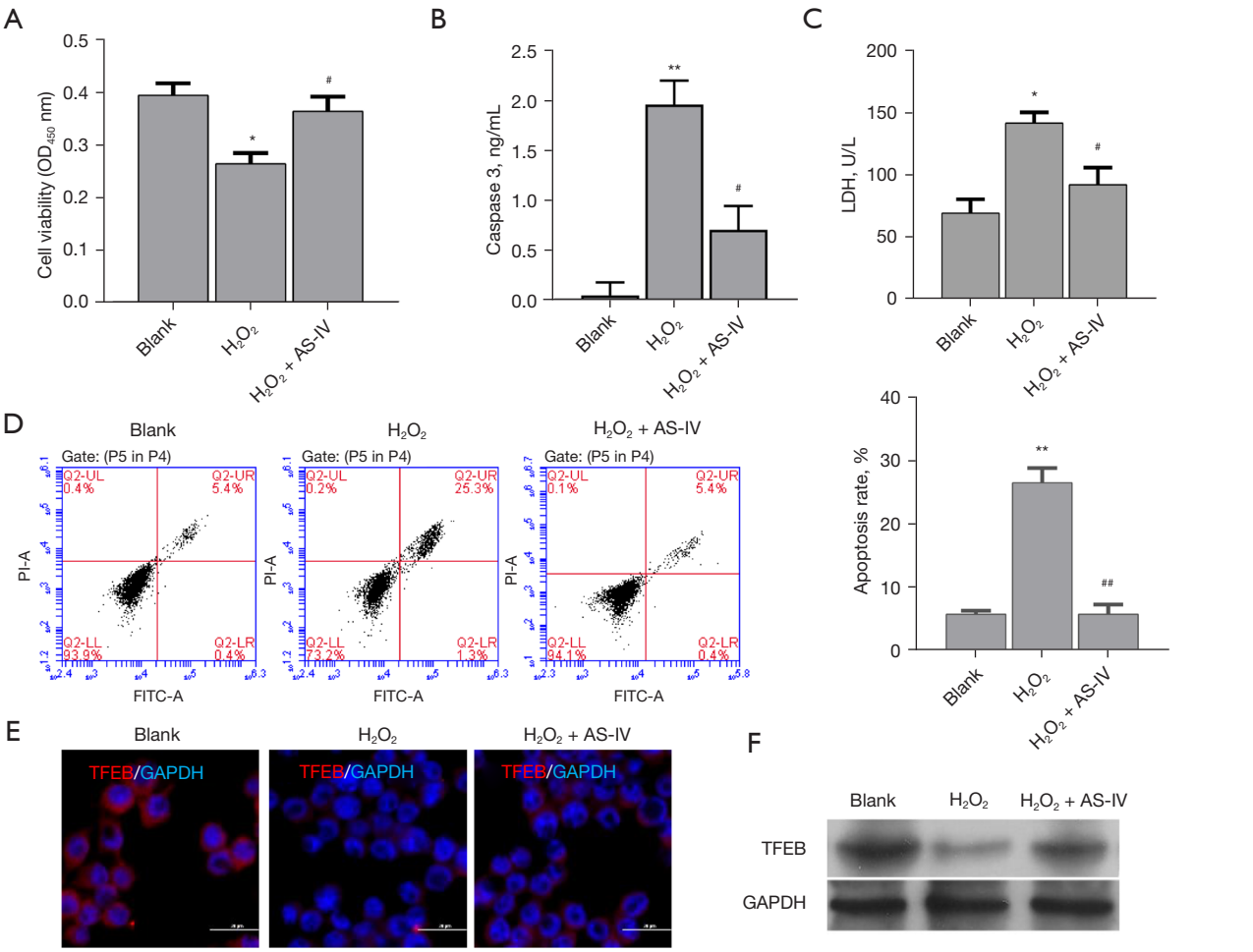

Figure 2 AS-IV protects PC12 cells against injury induced by H_2O_2 and enhances TFEB expression. PC12 cells were treated with 300 μmol/L H_2O_2, 0.1 μM AS-IV, and/or 5 μM FIN56. (A) The CCK-8 assay was used to determine the viability of PC12 cells; (B) Caspase-3 activity was measured to evaluate cell apoptosis; (C) ELISA was performed to verify the LDH concentration in PC12 cells; (D) flow cytometry was performed to determine the rate of apoptosis in PC12 cells. (E, F) TFEB expression was measured by using immunofluorescence (E) and Western blot (F), scale: 20 μm. *P<0.05, **P<0.01 *vs.* blank group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs.* H_2O_2 group; AS-IV; astragaloside IV; CCK-8, cell counting kit 8; ELISA, enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

treated cells (*Figure 3C*). To examine the role of ferroptosis in the protective effects of AS-IV in H_2O_2-treated PC12 cells, the expression of SLC7A11 and GPX4 was measured by Western blot analysis. As shown in *Figure 3D*, the expression of SLC7A11 and GPX4 was suppressed by H_2O_2 treatment, and recovered by AS-IV (*Figure 3D*). These results suggested that AS-IV exerted a protective action by inhibiting ferroptosis in H_2O_2-treated PC12 cells.

Verification of TFEB knockdown in PC12 cells

To further determine whether the protective action of AS-IV on SCI is associated with TFEB, PC12 cells were transfected with NC, si-TFEB#1, si-TFEB#2, or si-TFEB#3. The results of quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot verified that TFEB was dramatically downregulated in all cells

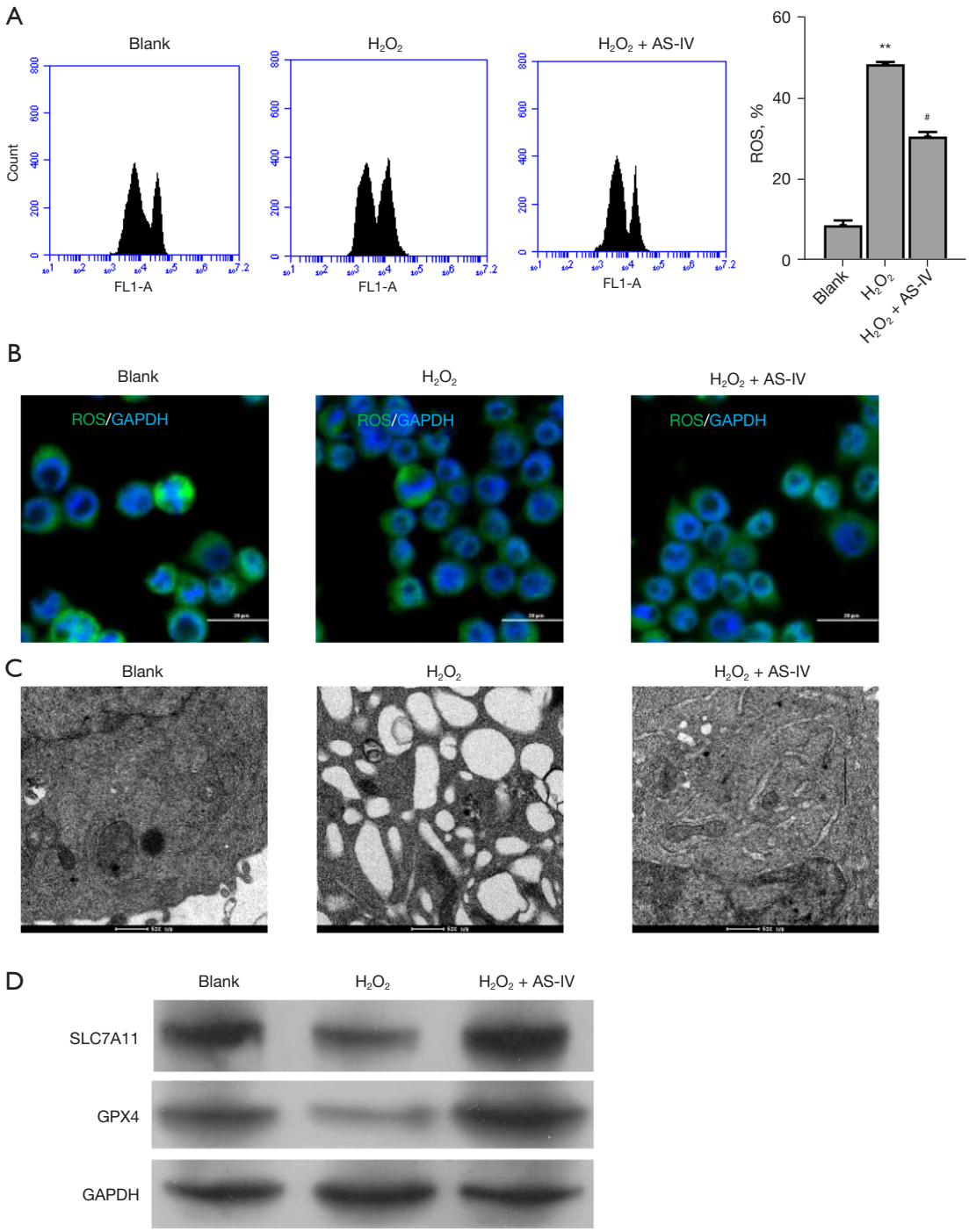

Figure 3 AS-IV inhibits ferroptosis in H_2O_2-damaged PC12 cells. PC12 cells were exposed to 300 μmol/L H_2O_2 and/or 0.1 μM AS-IV. (A) Flow cytometry was performed to quantify the levels of ROS in the cells. (B) DCFH-DA was used to monitor the levels of ROS in each group. Magnification, ×400; scale bar =20 μm. (C) The morphology of the mitochondria in each group of cells were observed using TEM. Scale bar =500 nm. (D)Western blot was used to confirm the expression of SLC7A11 and GPX4 in the treated PC12 cells. **P<0.01 *vs.* blank group; #P<0.05 *vs.* H_2O_2 group; AS-IV, astragaloside IV; ROS, reactive oxygen species; DCFH-DA, dichloro-dihydro-fluorescein diacetate; TEM, transmission electron micrograph; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SLC7A11, solute carrier family 7 member 11; GPX4, glutathione peroxidase 4.

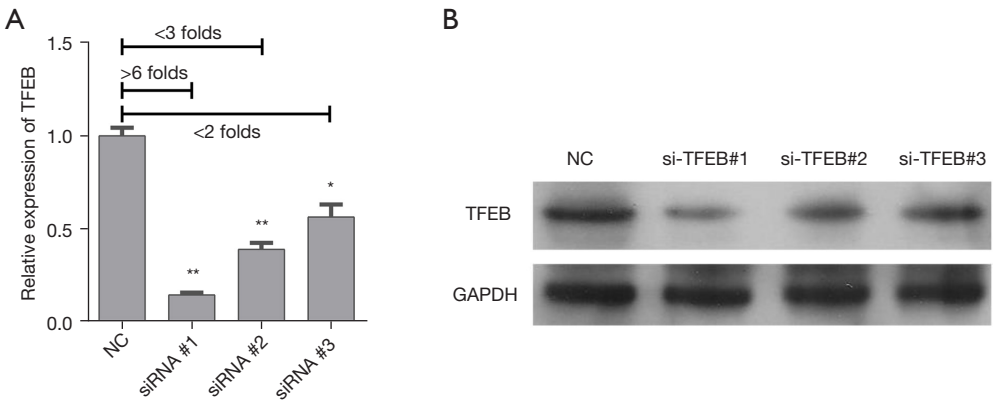

Figure 4 The effects of TFEB knockdown in PC12 cells. NC, si-TFEB#1, si-TFEB#2, and si-TFEB#3 were designed, synthesized, and transfected into PC12 cells. The transfection efficiency of the si-TFEBs were assessed by qRT-PCR (A) and Western blot (B). *P<0.05, **P<0.01 *vs.* NC group. TFEB, transcription factor EB; NC, negative control; si, small interfering; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transfected with the si-TFEBs compared to the NC group, in particular, si-TFEB#1 (*Figure 4A*,*4B*). Therefore, the si-TFEB#1 was used to transfect PC12 cells for all subsequent experiments.

TFEB-mediated ferroptosis is involved in H_2O_2-induced cell injury

We further explored whether the remission of H_2O_2-induced SCI by AS-IV is mediated through regulation of TFEB and ferroptosis. H_2O_2-treated PC12 cells were incubated with 0.1 μM AS-IV, si-TFEBs, and/or 5 μM FIN56. Knockdown of TFEB reversed the protective effect of AS-IV on cell viability in H_2O_2-treated PC12 cells, and FIN56 effectively enhanced the effect of si-TFEB (*Figure 5A*). Similarly, knockdown of TFEB reversed the protective effect of AS-IV on the rate of cell apoptosis in H_2O_2-treated PC12 cells. The combination of si-TFEB and FIN56 resulted in a further increase in the cell apoptosis rate compared with si-TFEB alone (*Figure 5B*). Furthermore, knockdown of TFEB was effective in ameliorating the decreased expression of caspase 3 and LDH caused by AS-IV in H_2O_2-treated PC12 cells. FIN56 further enhanced the effect of si-TFEB on caspase 3 expression in H_2O_2-treated PC12 cells treated with AS-IV (*Figure 5C*,*5D*). Immunofluorescence assays showed that TFEB expression was downregulated by TFEB siRNA, and its expression was further suppressed by the ferroptosis inducer, FIN56 (*Figure 5E*,*5F*). These results suggested that TFEB is required for the regulatory effects of AS-IV on proliferation and apoptosis of H_2O_2-damaged PC12 cells.

TFEB knockdown reversed the protective effect of AS-IV by mediating ferroptosis in H_2O_2-treated PC12 cells

The flow cytometry and DCFH-DA data demonstrated that AS-IV remarkably downregulated the levels of ROS, while TFEB silencing had the opposite effect by further increasing ROS accumulation. FIN56 enhanced the effects of si-TFEB (*Figure 6A*,*6B*). TEM observations revealed that si-TFEB reversed the protective effect of AS-IV on the mitochondria, and the addition of FIN56 further aggravated the mitochondrial abnormalities (*Figure 6C*). Transfection of si-TFEBs impaired the expression of SLC7A11 and GPX4, and this was further reduced by FIN56 (*Figure 6D*). Taken together, these results suggested that AS-IV alleviated H_2O_2-induced damage to PC12 cells through promoting TFEB expression and the subsequent suppression of ferroptosis.

Discussion

SCI is a common presentation for spinal surgery, and its pathological process includes primary and secondary injuries (28). Secondary injury is a series of physiological and biochemical reactions such as ischemia, edema, and electrolyte disturbance on the basis of the primary injury (29,30). Ischemia, hypoxia, and inflammation can cause the production and release of ROS (31-33), which can lead to oxidative damage of local tissues, as well as apoptosis and

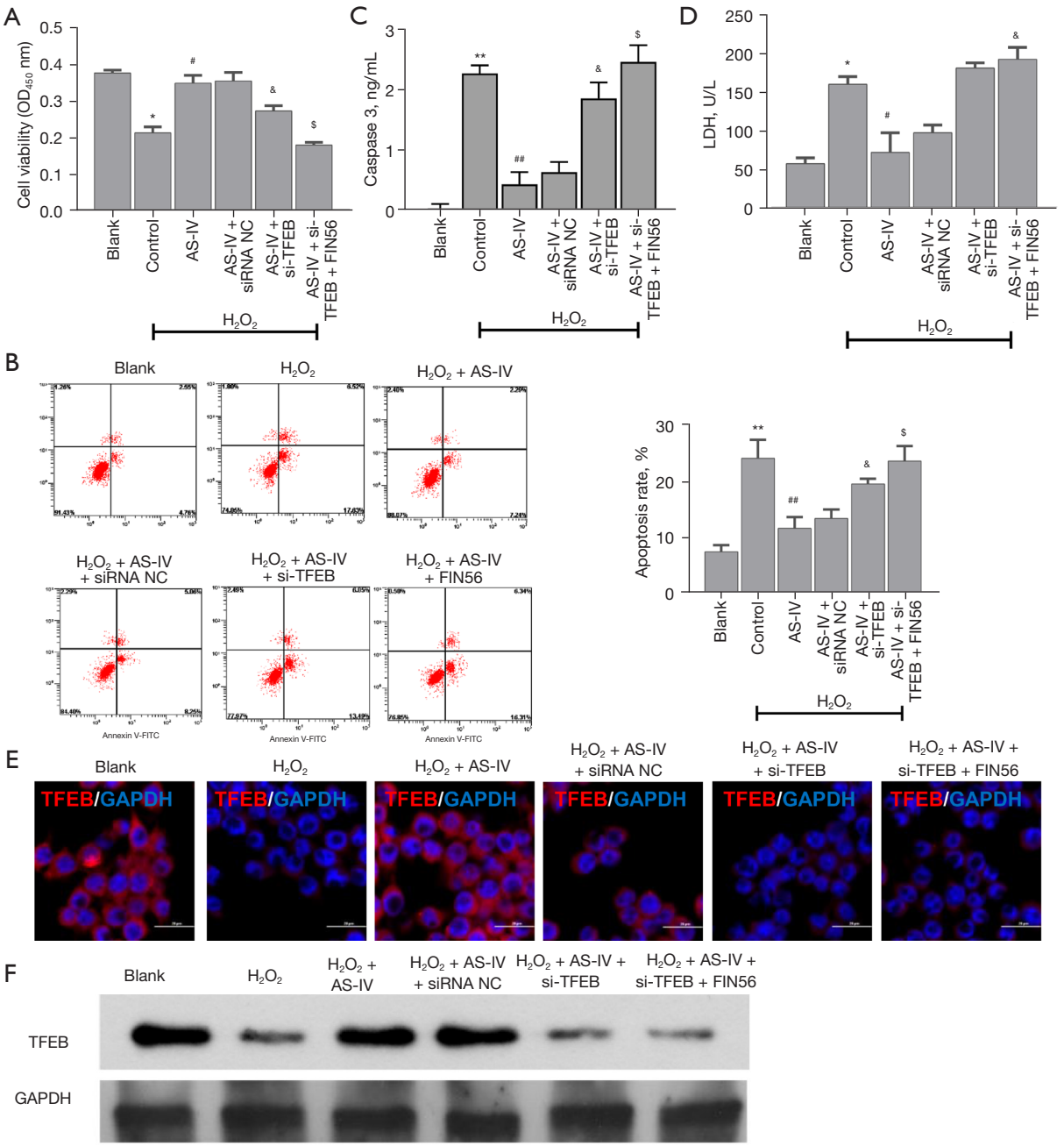

Figure 5 TFEB knockdown and ferroptosis promotion reversed the protective effect of AS-IV on cell injury induced by H_2O_2 in PC12 cells. H_2O_2-treated PC12 cells were incubated with 0.1 μM AS-IV, si-TFEBs, and/or 5 μM FIN56. (A) The viability of PC12 cells was monitored using the CCK-8 assay. (B) Flow cytometry was used to observe the changes in the rate of cell apoptosis in each group. (C) Caspase-3 activity was determined to analyze cell apoptosis. (D) The level of LDH was assess using ELISA. (E,F) IF assay (E) and Western blot (F) was conducted to determine the changes in TFEB expression in the nucleus and cytoplasm of PC12 cells. Magnification, ×400; scale bar =20 μm. *P<0.05, **P<0.01 *vs.* blank group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs.* H_2O_2 group; $^{\&}$P<0.05 *vs.* H_2O_2 + AS-IV + NC group; $^{\$}$P<0.05 *vs.* H_2O_2 + AS-IV + si-TFEB group. TFEB, transcription factor EB; AS-IV; astragaloside IV; si, small interfering; CCK-8, cell counting kit 8; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

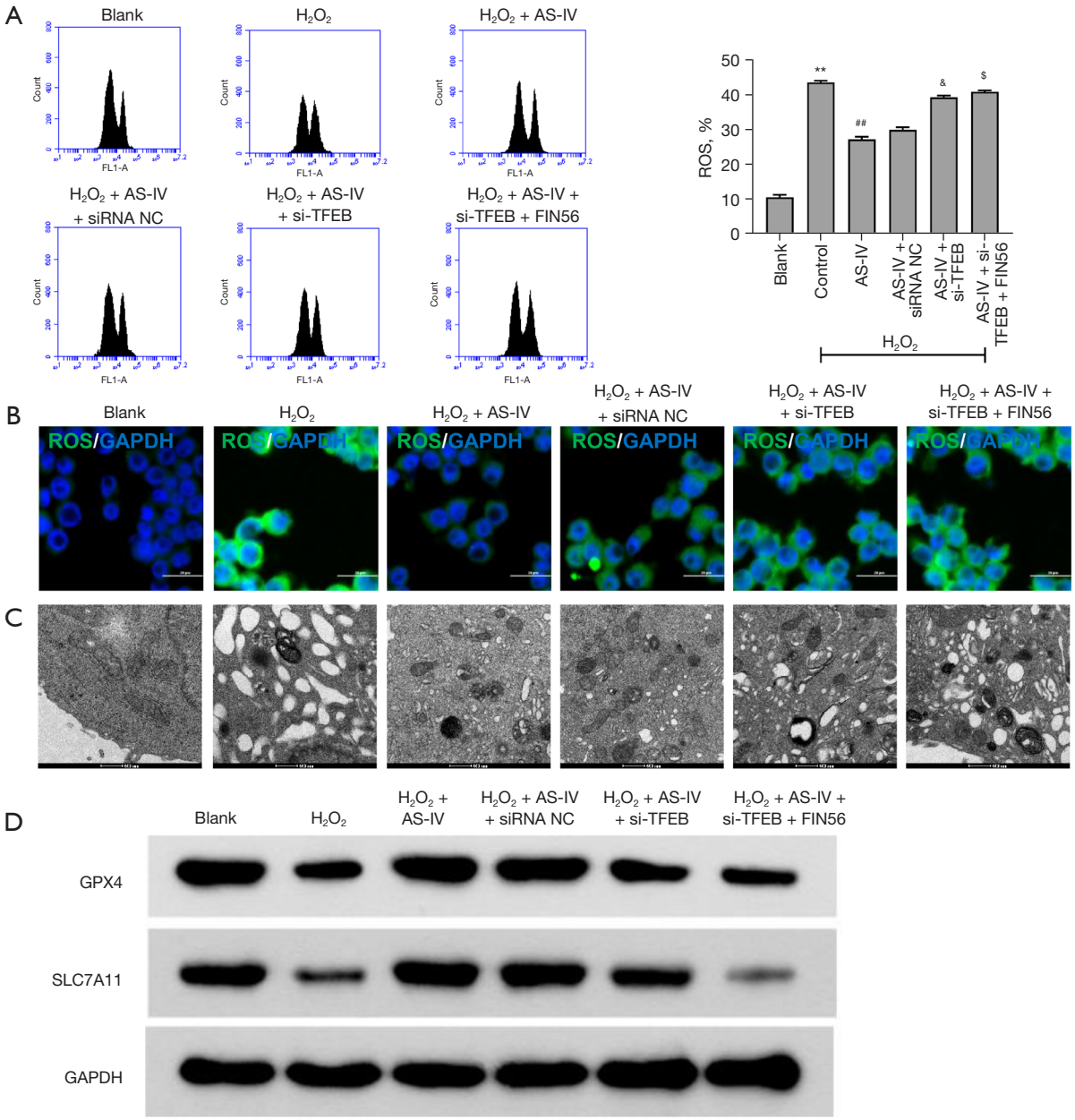

Figure 6 TFEB knockdown reversed the protective effect of AS-IV by mediating ferroptosis in H_2O_2-treated PC12. H_2O_2-damaged PC12 cells were treated with AS-IV, si-TFEBs, and/or FIN56. (A) Flow cytometry was performed to determine the rate of cell apoptosis in each group. (B) DCFH-DA was used to monitor ROS level in each group. Magnification, ×400; scale bar =20 μm. (C) TEM was used to observe the morphology of the mitochondria in each group. Scale bar =500 nm. (D) The levels of ferroptosis-associated proteins (SLC7A11 and GPX4) were measured by Western blotting analysis in each group. **P<0.01 *vs.* blank group; $^{\#\#}$P<0.01 *vs.* H_2O_2 group; $^{\&}$P<0.05 *vs.* H_2O_2 + AS-IV + NC group; $^{\$}$P<0.05 *vs.* H_2O_2 + AS-IV + si-TFEB group; AS-IV; astragaloside IV; ROS, reactive oxygen species; DCFH-DA, dichloro-dihydro-fluorescein diacetate; TEM, transmission electron micrograph; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SLC7A11, solute carrier family 7 member 11; GPX4, glutathione peroxidase 4.

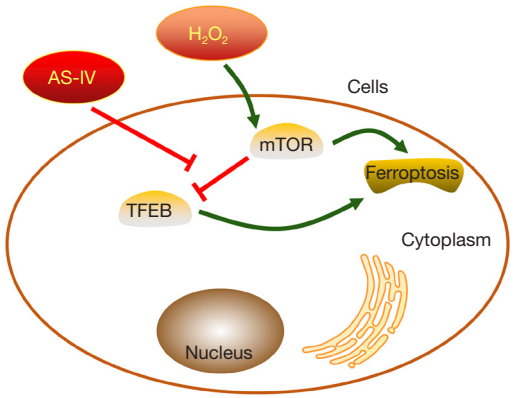

Figure 7 A graphic representation depicting the protective mechanism of AS-IV in H_2O_2-damaged cells. AS-IV, astragaloside IV; TFEB, transcription factor EB; mTOR, mammalian target of rapamycin.

necrosis of nerve cells (34). Therefore, the development of effective drugs to prevent the secondary death of spinal cord neurons is crucial.

Chinese herb derived active components, such as triptolide and salvianic acid B, have been proven to alleviate spinal cord injury through multiple pathways (35,36). AS-IV, as one of the main active components of Ast, has a wide range of pharmacological activities on the central nervous system (CNS) (12). Studies show that AS-IV has no obvious hepatotoxicity and nephrotoxicity (37,38). A large number of studies have shown that AS-IV can effectively delay the aging of body cells, promote proliferation, resist inflammation and apoptosis, and protect against oxidative stress of neuronal cells (15,39). In this current study, an oxidative stress injury model was established by pretreatment of PC12 cells with H_2O_2, and the effects of AS-IV were examined. The optimal concentration and exposure time of AS-IV in PC12 cells was determined to be 1.0 M and 48 h, respectively. AS-IV significantly enhanced proliferation, and inhibited apoptosis and ferroptosis in H_2O_2-damaged PC12 cells, and this effect was reversed by FIN56. This suggested that the ferroptosis pathway is involved in the protective effect of AS-IV on SCI.

Studies have demonstrated that ferroptosis signaling seriously affects SCI. After SCI, massive hemorrhage, cell rupture, hemolysis, erythrocyte aggregation, and iron overload occur in the spinal cord, and stress increases the production of ROS and the excitotoxicity of glutamate (40,41). All these factors can stimulate ferroptosis (42). Some ferroptosis inhibitors, such as SRS 16-86 (43), lipro-1 (44), and deferoxamine (40) may be potential drugs for the treatment of SCI. FIN56 is a ferroptosis inducer and triggers ferroptosis via inducing the degradation of GPX4 (45). Some reports have shown that ferroptosis is closely related to lysosomal membrane permeabilization (46). The microphthalmia-associated transcription factor (MITF) family include MITF, TFEB, TFE3 and transcription factor EC (TFCE) (47). Among them, TFEB can be expressed in a variety of tissues (48). Since TFEB is reported to be essential in lysosomal biogenesis (49) and clearance of oxidative stress (50), we hypothesized that TFEB might participate in the ferroptosis process of SCI.

The current investigation demonstrated that AS-IV notably promoted the expression of TFEB in H_2O_2-damaged PC12 cells. In addition, TFEB was involved in the regulatory effects of AS-IV on the proliferation and apoptosis of H_2O_2-treated PC12 cells. Moreover, AS-IV-mediated expression of TFEB was remarkably attenuated by TFEB knockdown, and the combination of FIN56 and si-TFEB further attenuated the effects of AS-IV on H_2O_2-damaged PC12 cells (*Figure* 7).

Conclusions

AS-IV can protect PC12 cells against oxidative injury mediated by H_2O_2 via expression of TFEB and the subsequent suppression of ferroptosis. AS-IV is expected to inhibit the apoptosis of nerve cells caused by spinal cord injury and induce the generation of spinal cord scar. At present, the clinical development and promotion of AS-IV has been stagnant due to the low output of astragalus and the difficulty in chemical synthesis. Therefore, there is still a lack of corresponding clinical data support. The researchers believe that AS-IV derivatives with high water solubility and bioavailability should be concerned. While, this requires more in-depth research, development and utilization.

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Footnote

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