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Overexpression of FSP1 Ameliorates ferroptosis via PI3K/ AKT /GSK3β pathway in PC12 cells with Oxygen-Glucose Deprivation/ Reoxygenation

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ABSTRACT

After ischemia and reperfusion (I/R), nerve cell damage is a pathogenic process that involves numerous molecular processes. In the last ten years, one new classification of programmed cell death is ferroptosis. More recent research has demonstrated that ferroptosis has a role in a variety of neurological disorders, including stroke, cancer, and neurodegenerative illnesses. Ferroptosis suppressor protein 1 (FSP1) plays a significant role in inhibiting ferroptosis. The purpose of this work is to determine how overexpression of FSP1 affects the ferroptosis of PC12 cells under the condition of oxygen-glucose deprivation/reoxygenation (OGD/R). The expression of FSP1 was regulated by lentivirus transfection technology. Western blot and immunofluorescence were used to measure protein levels related to ferroptosis and the PI3K/AKT/GSK3β signal pathway. Determine cell viability using the appropriate kit. Mitochondrial structural morphology was checked by transmission electron microscopy in PC12 cells. Reactive oxygen species (ROS) and Malondialdehyde (MDA) were quantified using the relevant kits. OGD/R induced ferroptosis in PC12 cells, however, FSP1 overexpression reverses ferroptosis and promotes cell viability, lowering ROS and MDA content. The expression of FSP1 decreased in OGD/R0h and OGD/R6h and rebounded in OGD/R24h and OGD/R48h. During the processes of OGD/R-induced ferroptosis, FSP1 overexpression significantly stimulated PI3K/AKT/GSK3β pathway, but LY294002 weakens the protective effect of FSP1 overexpression. Our outcomes demonstrate that overexpression of FSP1 markedly enhances the ability to resist ferroptosis via the PI3K/AKT/GSK3β pathway. The above results may provide a new preliminary lead for the treatment of the cerebral ischemia-reperfusion injury.

1. Introduction

Ischemic stroke is an issue that affects patients' survival and quality of life on a global scale [1]. Different treatment methods and time points may lead to great differences in clinical outcomes, recanalization is an important first-line treatment [2]. However,

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ischemia and reperfusion (I/R) injury caused by vascular recanalization is still a key factor affecting the prognosis of ischemic stroke [3]. A series of cell biochemical and pathological changes lead to nerve cell death during ischemia and reperfusion. Rescuing nerve cell death by I/R remains a key priority in the clinic, although therapeutic options are limited. Including necrosis, apoptosis, autophagy, and other programmed cell death (PCD), these have been a question of intense discussion. By contrast, ferroptosis differs from classical types of PCD, and has specific morphological changes with mitochondrial shrinkage, fragmentation, and decreased mitochondrial cristae. Earlier studies have noted that stroke can lead to a build-up of iron in cells and increase neuronal damage in animals and humans [4,5].

Several studies have shown that iron chelators attenuate brain damage from ischemic stroke in animal models [6]. Fer -1, a ferroptosis inhibitor, has a protective effect on neuronal damage in MCAO mouse models [7]. These studies suggest that ferroptosis plays a key role in brain I/R injury and that inhibition of ferroptosis can protect against I/R-induced brain tissue damage. However, the specific role and mechanism of ferroptosis in cerebral ischemia-reperfusion injury are still poorly understood. Recent years, ferroptosis, an iron-dependent programmed cell death that accumulates in lipid peroxidation, has attracted increasing attention from researchers [8–11]. Glutathione peroxidase 4 (GPX4) has been recognized as an important regulator of a canonical axis of the Cyst(e)ine/glutathione/GPX4 [12]. Ferritin heavy chain 1 (FTH1) is considered a ferroptosis marker protein [13], which is ubiquitously expressed in different tissues and cell lines. Inducers of ferroptosis, such as erastin, work on the X^{c-}signaling pathway [8]. Accumulation of MDA and ROS is also characteristic of ferroptosis. Interestingly, in addition to GPX4, a protein called ferroptosis suppressor protein 1 (FSP1) was discovered to prevent ferroptosis. [14,15]. Firstly, FSP1 was discovered as a responsible gene for P53 [16] and has the role of caspase-independent induction of apoptosis. Although more research is still needed on the underlying mechanisms. Most recent studies proposed that FSP1 has nicotinamide adenine dinucleotide oxidase activity, as a member of the nicotinamide adenine dinucleotide family, and can reduce phospholipid peroxidation via lowering the level of oxidized coenzyme Q10 (CoQ10) to abrogate ferroptosis [14,17–19]. Coenzyme Q10 (CoQ10) is crucial for the proper physiological operation of the electron transport chain and Adenosine triphosphate generation, but it also reduces ROS production through the PI3K/AKT pathway [20]. The activity of the PI3K/AKT pathway has a positive influence on neuronal survival after trauma and neurological deficits [21,22]. In addition, decreased activity of GSK-3β can enhance cellular antioxidant defense by activating nuclear factor E2-related factor 2 [23]. Based on the aforementioned research, ferroptosis and ischemia-reperfusion damage are intimately associated with the PI3K/AKT/GSK-3β pathways. However, there is currently a lack of research on this topic. This field may be relatively novel, but there is not enough research on it, and more in-depth research and exploration are needed.

It is fair to believe that FSP1 may act through the PI3K/AKT/GSK-3β pathway to prevent ferroptosis. The current study examines oxygen-glucose deprivation/reperfusion (OGD/R) triggers ferroptosis in PC12 cells and how FSP1-overexpression might mitigate this effect. OGD/R is a widely used experimental model to mimic ischemic stroke *in vitro*. The selection of OGD/R conditions and the mimicry of the stroke microenvironment are important factors to consider in order to improve the relevance and translational potential of the model. FSP1 protects PC12 cells from OGD/R-induced ferroptosis partly by PI3K/AKT/GSK-3β signaling pathway. Therefore, ferroptosis is a critical reason for cell death related to cerebral ischemia-reperfusion injury. This research provides a new theoretical basis for further understanding the pathogenesis of neurological damage in ischemic stroke.

2. Materials and methods

2.1. Chemicals

Erastin was purchased from Selleck (CAS: 571203-78-6, Shanghai, China). LY294002 (PI3K inhibitor) was provided by Beyotime Biotechnology (S1737-1 mg, Shanghai, China).

2.2. PC12 cells cultures

Procell Life Science &Technology Co. Ltd. (Wuhan, China) graciously contributed the PC12 cells that were used to determine the species. Thermo Fisher Scientific provided all materials pertaining to cell cultures (Waltham, MA, USA). Nerve growth factor (NGF) (50 ng/ml) [24] was used to stimulate cell development, and cells were then maintained at 36.5 °C ± 0.5 °C in an environment of 95% air and 5% CO2. All experimental groups of cells were cultured in medium (DMEM+10% fetal bovine serum). The medium was replaced every 36 h. Cells were used in the experiments after 3–4 d.

2.3. OGD/R and drug treatment

To cause OGD damage, cells were grown in glucose-free DMEM and exposed to an anoxic chamber with 94% N_2, 5% CO_2, 1% O_2 and for 4 h. Following OGD, the medium was changed to a brand-new neurobasal media, and cells were incubated for 6 h at under normal conditions. For the same amount of time, control cells were cultured in glucose-containing medium in an incubator with normoxic conditions. We established drug groups with different concentrations in pre-experiments and screened out the best application concentration. According to the manufacturer's instructions, a 10 mM erastin stock solution was prepared, then dilute the stock solution to a 10 μM solution with the basal medium. LY294002 was diluted as a 30 μM solution for pretreatment [25]. Then the erastin group were replaced with medium supplemented with 10 μM erastin, and continued to culture in the incubator for 6 h. LY294002 was independently added for 1 h prior to treatment of OGD/R. LY294002 is a specific inhibitor of PI3K. We used LY294002 to demonstrate PI3K/AKT/GSK-3β pathway was crucial to resist ferroptosis during cerebral ischemia-reperfusion injury.

2.4. Measuring the levels of ROS inside cells

ROS Assay Kit was utilized so that levels of intracellular ROS could be determined (S0033S, Beyotime Biotechnology, Shanghai, China). After diluting DCFH-DA with serum-free culture media at a ratio of 1:1000 to reach a final concentration of 10 μmol/L, the cells were stained with DCFH-DA for a period of 20 min in the absence of light. The microplate reader was utilized in order to evaluate the fluorescence intensity. Every experiment was performed three times, and each time it was measured.

2.5. Measuring the levels of MDA inside cells

Utilizing the Lipid Peroxidation MDA Assay Kit (S0131S, Beyotime Biotechnology Shanghai, China), MDA levels were assessed. We created blank control, standard, and sample groups as per the kit's instructions, then added 0.1 ml of test samples to the sample group, 0.1 ml of PBS to the control group, and 0.1 ml of standards to the standard group. In each group, add 0.2 ml of the MDA detection working solution. After cooling to room temperature and centrifuging for 10 min, the combined liquid was heated in a boiling water bath for 15 min. A precise reading of the absorbance of the sample is obtained by using the fluorescent enzyme marker between 530 and 540 nm. Every experiment was measured three times.

2.6. Measurement of intracellular iron

Iron Colorimetric Assay Kit was utilized so that levels of intracellular iron could be determined (E1042-100, Applygen, Beijing, China). First, we collected each group of experimental cells and extracted cell lysates. Then, we created blank control, standard, and sample groups as per the kit's instructions, then added 0.1 ml of test samples to the sample group, 0.1 ml of the mixture A to the control group, and 0.1 ml of standards to the standard group. After incubating at 60 °C for 1 h, add 30 μl of working solution and incubate at room temperature for 30 min. Finally, the absorbance value was measured at 550 nm. Every experiment was measured three times.

2.7. Measurement of cell viability

For cell viability analysis, cells were seeded in a 96-well plate at the cell densities of 1×10^5 cells per well. Using the Cell Counting Kit-8 assay and Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit, cellular proliferation was discovered. Both the Cell Counting Kit-8 assay kit (C0037, Beyotime Biotechnology Shanghai, China) and the Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit (C2015 M, Beyotime Biotechnology Shanghai, China) were used in the experiment as directed by the kit's instructions. Every experiment was measured three times.

2.8. Immunofluorescence

After being fixed for 20 min in 4% paraformaldehyde, the cells were allowed to recover by being permeabilized with 0.3% Triton ×100. Following this, the cells were allowed to remain in the immunol staining blocking buffer for one additional hour. FSP1 primary antibody was then treated with cells for an overnight period at 4 °C. Cells were treated with a secondary antibody that had been Alexa fluor 488-tagged for 2 h at room temperature. Between each stage, wash three times with PBS.

2.9. Western blots analysis

The list of primary antibodies is as follows: FSP1 (20886-1, Proteintech, Wuhan, China), GPX4 (67763, Proteintech, Wuhan, China), FTH1 (381204, Zen BioScience, Chengdu, China), β-tubulin (10068, Proteintech, Wuhan, China), PI3K (20584, Proteintech, Wuhan, China), p-PI3K (AF3242, Affinity Biosciences, Jiangsu, China), p-AKT (9018S, Affinity Biosciences, Jiangsu, China), AKT (4691S, Cell Signaling Technology, USA), p-GSK3β (14850, Proteintech, Wuhan, China), GSK3β (22104, Proteintech, Wuhan, China). After cell harvesting, extract the protein as quickly as you can. Use BCA method to detect protein content of samples. By using gel electrophoresis, the samples (20 g per lane) were separated from the target proteins, and after that, the proteins were blotted onto a PVDF membrane. After being blocked with 10% non-fat milk powder at room temperature for a duration of 2 h, the membranes were then incubated with the primary antibodies for a period of one night at a temperature of 4° Celsius. The following day, the membranes were incubated with secondary antibodies that matched the primary ones for 2 h at room temperature.

2.10. Transmission electron microscopy (TEM)

Briefly, after trypsinizing, the cells were centrifuged and harvested. The 3%glutaraldehyde with 0.1 mol/L PBS (1:1) was then added, and cells were resuspended and allowed to stand for 5 min at 4 °C. The cell suspension was further centrifuged at high speed (12000 rpm, 10 min), and the precipitate was retained. Finally, glutaraldehyde was used to fix the precipitate. Subsequently, samples were observed under the TEM.

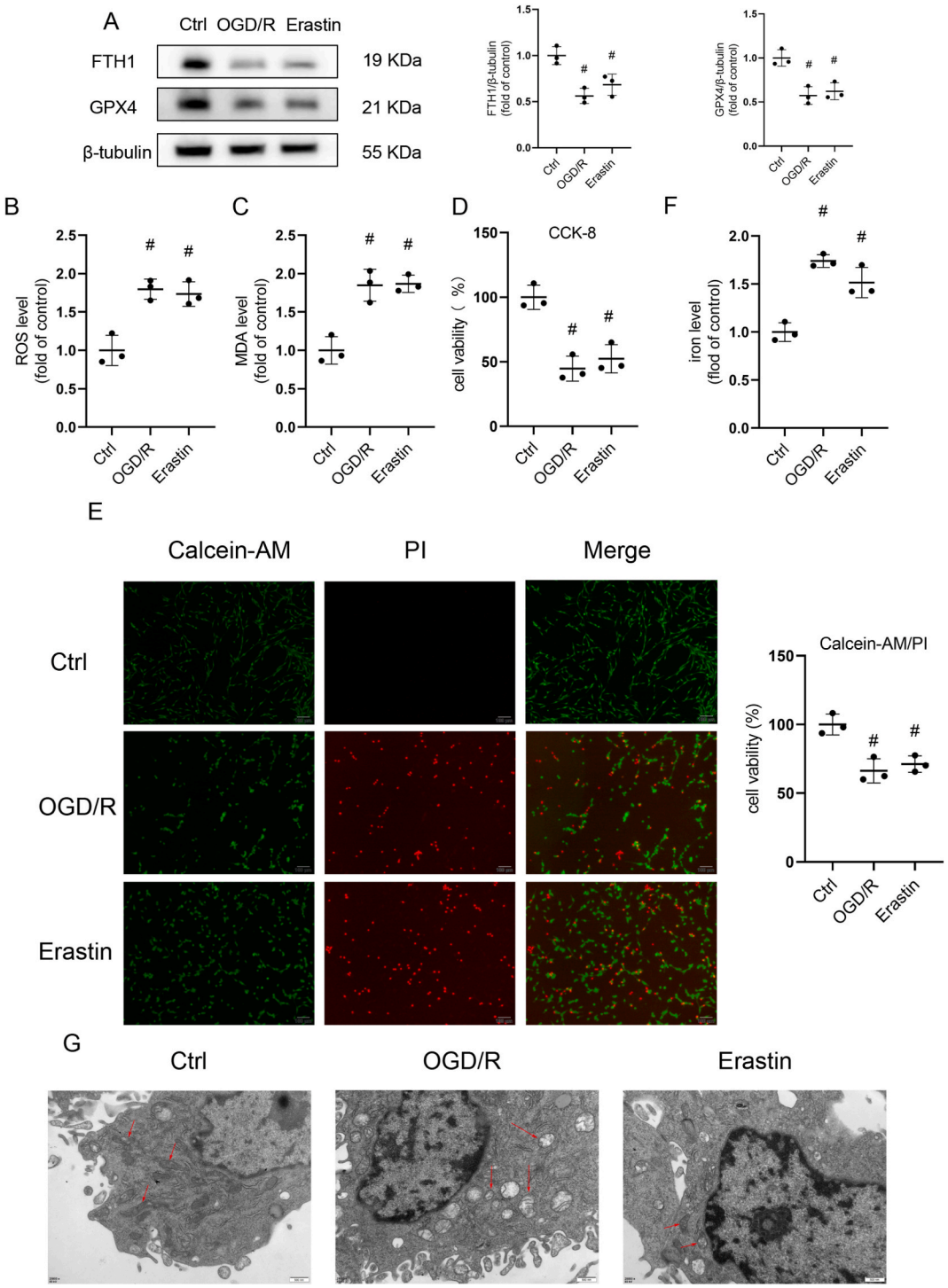

Fig. 1. OGD/R induces ferroptosis in PC12 cells. (A) PC12 cells were untreated, stimulated with OGD/R, or pretreated with Erastin (10 μM). Then, Western blot detected FTH1 and GPX4 protein levels and showed that OGD/R or Erastin reduced protein expression. (B) ROS assay showed that OGD/R or Erastin increased ROS level compared to the control. (C) Lipid Peroxidation MDA Assay showed that OGD/R or Erastin increased MDA levels compared to the control. (D) CCK-8 assay showed that OGD/R or Erastin increased cell death compared to the control. (E) Typical pictures of Calcein-AM/PI staining showed that OGD/R or Erastin increased cell death compared to control. The scale bar is 100 μm. (F) Compared with the control group, both OGD/R and erastin treatment increased iron levels. (G) Typical pictures of mitochondrial morphology showed that OGD/R or Erastin induced mitochondrial morphological changes in ferroptosis compared to control. The scale bar is 500 nm. The data were represented as means ± SD, n = 3, $^{\#}p < 0.05$ vs. control group.

2.11. Viral vectors

BrainVTA created the FSP1 overexpression lentiviral vector, the FSP1 expression interference vector, and their respective control vectors (BrainVTA Co., Ltd., Wuhan, China). Following digestion and seeding of the cell lines into plates, they were transfected with the designated vectors. The regulatory sequence of the FSP1 gene is as follows: mRNA NCBI reference：NM_144753.2

sh-NC：5'-GCCTAAGGTTAAGTCGCCCTC-3'

shRNA-FSP1：5'-GCCCCTGCTGATCAACATGTA-3'.

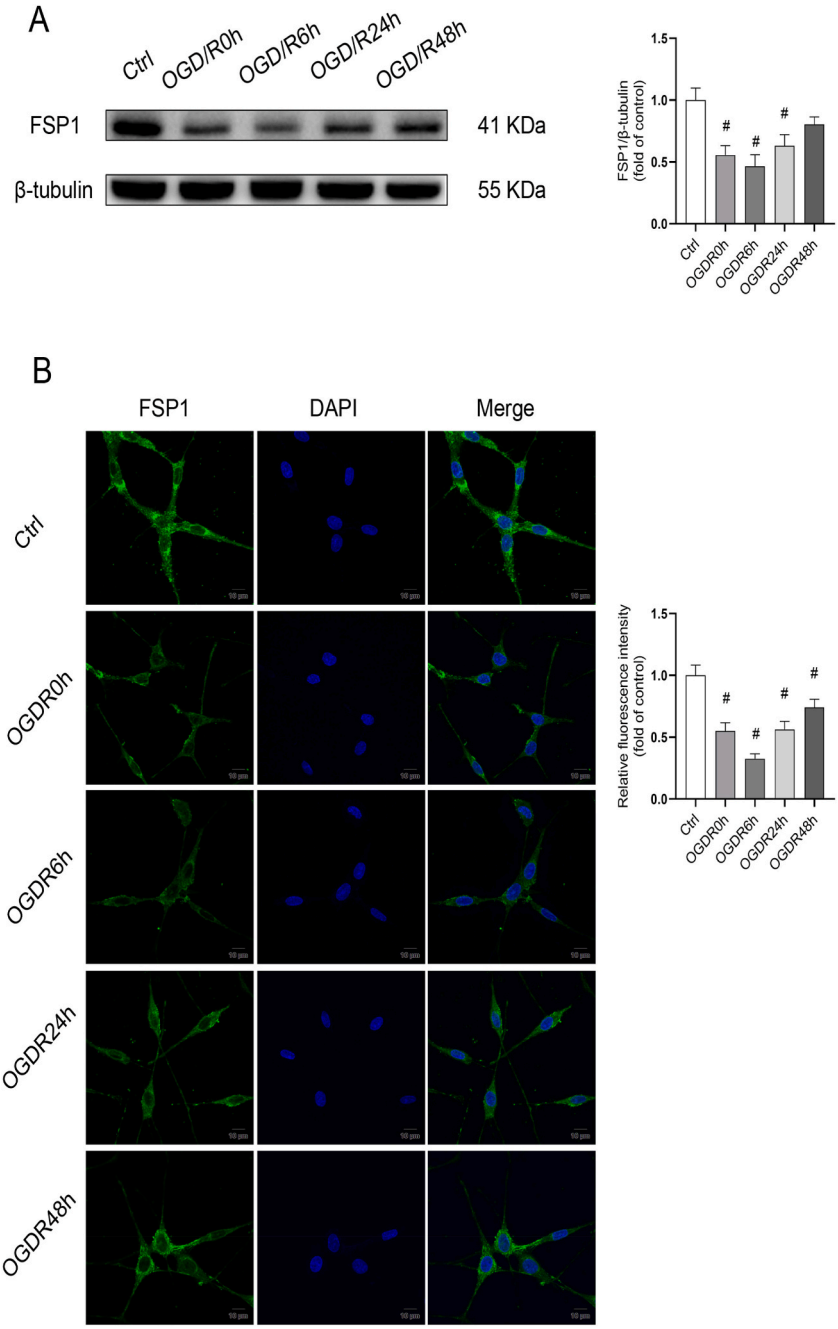

Fig. 2. The level change of FSP1 in PC12 cells with OGD/R. (A) Representative images of Western blotting of FSP1 in each group with untreated or OGD/R. OGD/R0h, OGD/R6h, and OGD/R24h, but not OGR/R48h, decreased expression of FSP1 compared to control. (B) Typical immunofluorescence pictures show that OGD/R0h, OGD/R6h, OGD/R24h, and OGR/R48h decreased expression of FSP1 compared to the control. The scale bar is 10 μm. The data were represented as means ± SD, n = 3, $^{\#}p < 0.05$ vs. control group.

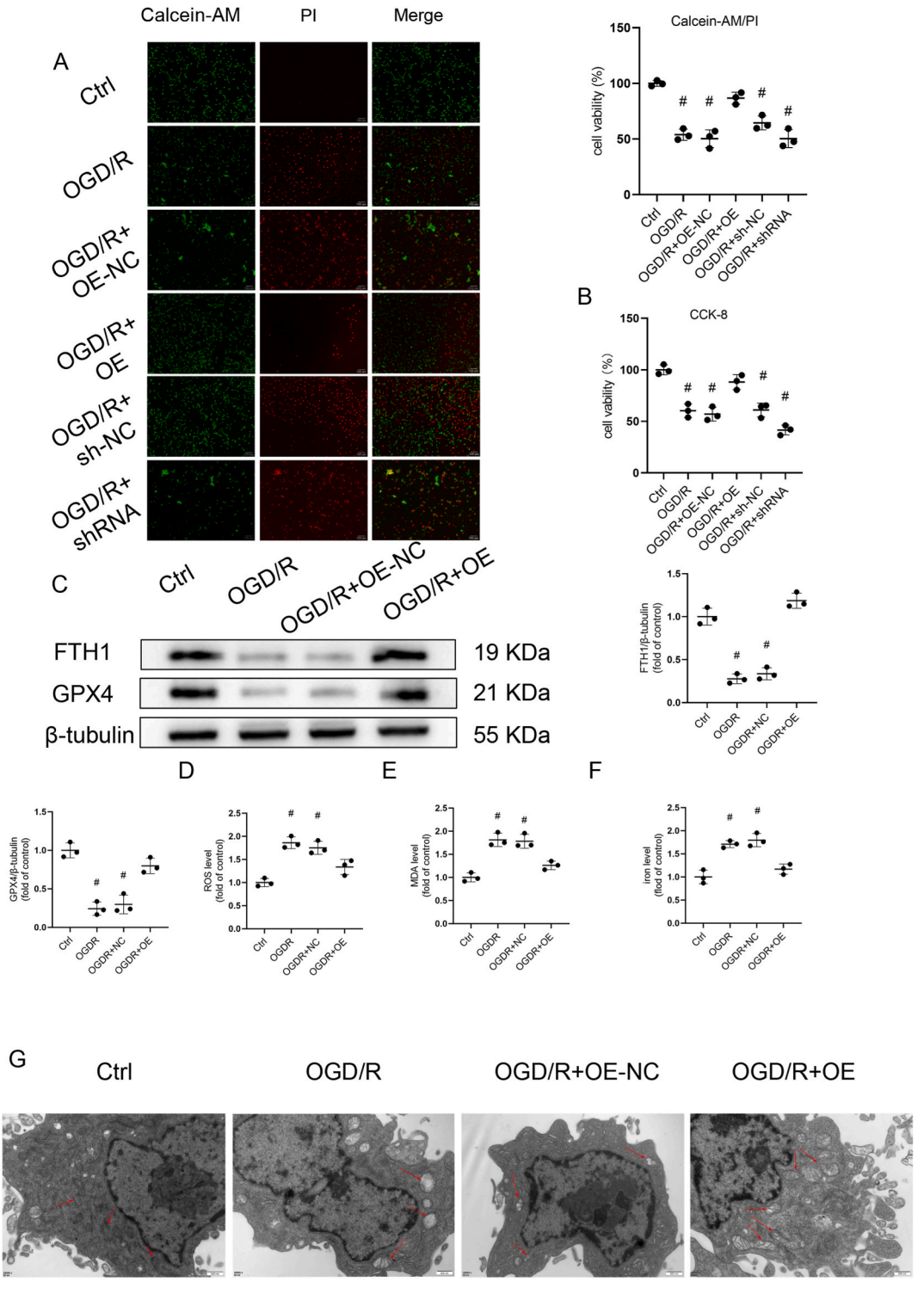

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Fig. 3. Overexpression of FSP1 attenuates ferroptosis following OGD/R in PC12 cells. (A) Typical pictures of Calcein-AM/PI staining showed that OGD/R, OGD/R + OE-NC, OGD/R + sh-NC, and OGD/R + shRNA increased cell death compared to the control group. The scale bar is 100 μm. (B) CCK-8 assay showed that OGD/R, OGD/R + OE-NC, OGD/R + sh-NC, and OGD/R + shRNA increased cell death compared to the control group or OGD/R + OE group. (C) Representative images of Western blotting of FTH1 and GPX4 in each group. The OGD/R and OGD/R + OE-NC decreased FTH1 and GPX4 levels compared to the control group. (D) ROS assay showed that OGD/R and OGD/R + OE-NC increased ROS levels compared to the control group. (E) Lipid Peroxidation MDA Assay showed that OGD/R and OGD/R + OE-NC increased MDA levels compared to the control group. (F) Compared with the control group, iron levels increased in groups OGD/R and OGD/R + OE-NC. (G) Typical pictures of mitochondrial morphology show that Overexpression of FSP1 attenuates mitochondrial morphological changes compared to the OGD/R group and OGD/R + OE-NC group. The scale bar is 500 nm. Values are means ± SD of data, n = 3, $^{\#}p < 0.05$ vs. control.

2.12. Statistical analysis

A statistical analysis program called GraphPad Prism 8.0.2 was used. The data were broken down and provided with their mean value as well as their standard deviation (mean ± SD). The normality and variance homogeneity of data was assessed by the Shapiro-Wilk normality test and Brown-Forsythe before analysis. One-way ANOVA followed by Tukey's post hoc test was utilized for comparisons between multiple groups. The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1. Ferroptosis does occur in PC12 cells with OGD/R treatment

Various mechanisms have been confirmed to be involved in cerebral ischemia-reperfusion injury, and our data shows that ferroptosis does occur. We investigated protein levels of GPX4 and FTH1 by Western blots, and these are essential proteins for regulating ferroptosis. As shown in Fig. 1A, the GPX4 and FTH1 protein expression of the OGD/R group and the erastin group (10 μM) was significantly decreased compared with the control group ($p < 0.05$). We then detected the effects of OGD/R on ROS and MDA activity (Fig. 1B and C). Compared with the control group, OGD/R increased ROS (179.7%) and MDA (184.9%) activity ($p < 0.05$). Compared with the control group, erastin increased ROS (173.5%) and MDA (186.8%) activity ($p < 0.05$). Next, the results of the CCK-8 assay and Calcein/PI staining for cell viability are shown in Fig. 1D and E. Both OGD/R and erastin treatment caused cell damage ($p < 0.05$). Compared with the control group in cell viability, OGD/R caused a 55.25% decrease by CCK-8 assay, a 33.7% decrease by Calcein/PI staining. Erastin caused a 47.59% decrease by CCK-8 assay, a 28.83% decrease by Calcein/PI staining. We then measured the levels of intracellular iron in each group (Fig. 1F). Compared with the control group, both OGD/R and erastin treatment increased iron levels to 174% and 151.6% ($p < 0.05$). Finally, changes in mitochondrial morphology further confirm the existence of ferroptosis (Fig. 1G). Mitochondria get shorter and thicker, and mitochondrial cristae decrease or disappear in OGD/R and erastin groups. These remarkable phenomena were observed under transmission electron microscopy. These results suggested the effect of OGD/R on ferroptosis.

3.2. Changes in expression of FSP1 in PC12 cells after OGD/R treatment

In the past several years, further research suggested that there may be other undiscovered vital mechanisms involved in ferroptosis. Previous studies have shown that FSP1 may play a protective role as a new target against ferroptosis. However, we know very little about it in cerebral ischemia and reperfusion injury. As shown in Fig. 2A, in contrast to the expression level of FSP1 in the control group, decreased protein level of FSP1 in OGD/R group ($p < 0.05$). Although expression levels in OGD/R24h (64.25%) and OGD/R48h (80.46%) are increased relative to OGD/R0h (55.62%) and OGD/R6h (46.56%). FSP1 expression was further assessed by immunofluorescence, and results showed a similar trend (Fig. 2B). Compared with the control group, the expression of FSP1 decreased by 44.88% in OGD/R0h group, 67.42% in OGD/R6h group, 43.7% in OGD/R24h group, and 25.76% in OGD/R48h group ($p < 0.05$).

3.3. The effect of FSP1-overexpression to resist ferroptosis in PC12 cell with OGD/R treatment

In order to gain a better understanding of the role that FSP1 plays in the cell damage caused by OGD/R, we use lentiviral transfection technology to regulate the expression of FSP1 (Detailed data can be found in supplementary materials). We then detect cell viability in each group (Fig. 3A and B). The Calcein-AM/PI staining results showed that compared to the control group, the cell viability of OGD/R group, OGD/R + OE-NC group, OGD/R + sh-NC group, and OGD/R + shRNA group decreased by 46.1%, 49.7%, 35.5%, and 49.67%, respectively ($p < 0.05$), and the cell viability of OGD/R + OE group decreased by 13.23% ($p = 0.1621$). The CCK-8 assay results showed that compared to the control group, the cell viability of OGD/R group, OGD/R + OE-NC group, OGD/R + sh-NC group, and OGD/R + shRNA group decreased by 39.67%, 42.98%, 38.93%, and 58.44%, respectively ($p < 0.05$), and the cell viability of OGD/R + OE group decreased by 11.84% ($p = 0.2531$). Next, FTH1 and GPX4 protein levels were evaluated (Fig. 3C). The GPX4 and FTH1 expression were increased in OGD/R + OE group ($p < 0.05$, vs. control).

Furthermore, we investigated ROS and MDA levels. Similarly, overexpression of FSP1 was able to decrease ROS and MDA levels augmented by OGD/R treatment (Fig. 3D and E). Compared with the control group, ROS levels increased to 186.2% and 175.2% in groups OGD/R and OGD/R + OE-NC ($p < 0.05$, vs. control), respectively, and ROS levels rose to 133.7% in group OGD/R + OE ($p = 0.0589$, vs. control). Compared with the control group, MDA levels increased to 181.3% and 178.2% in groups OGD/R and OGD/R + OE-NC ($p < 0.05$, vs. control), respectively, and MDA levels rose to 126.1% in group OGD/R + OE ($p = 0.1225$, vs. control). We then

measured the levels of intracellular iron in each group (Fig. 3F). Compared with the control group, iron levels increased to 171% and 179.7% in groups OGD/R and OGD/R + OE-NC ($p < 0.05$, vs. control), respectively. However, iron level rose to 117% in group OGD/R + OE ($p = 0.3862$, vs. control). Meanwhile, overexpression FSP1 rescued mitochondrial pathological changes in ferroptosis (Fig. 3G). Overall, these results confirm that overexpression of FSP1 can resist ferroptosis and reduce cell death with OGD/R insult.

3.4. The FSP1-overexpression promotes the p-PI3K, p-AKT, and p-GSK3β in PC12 cells with OGD/R

Given that FSP1 overexpression against ferroptosis by OGD/R, potential pathways need more attention. We selected cells that were deprived of oxygen and glucose for 4 h and reoxygenated for 6 h for the experiment. Interestingly, an experimental result about this was observed. Fig. 4 shows the FSP1-overexpression group with OGD/R treatment exhibited higher p-PI3K, p-AKT, and p-GSK3β protein levels ($p < 0.05$, vs. control).

3.5. The protective effects of overexpression FSP1 are mediated via the activation of PI3K

In order to determine whether or not the PI3K/AKT/GSK3β pathway is involved in the protective effect of FSP1 overexpression against OGD/R-induced damage. Cells were pretreated with PI3K inhibitors LY294002 for 1 h and then treated with OGD/R. Subsequently, cell survival was assessed by Calcein-AM/PI staining and CCK-8 assay (Fig. 5A and B). The Calcein-AM/PI staining results showed that compared to the control group, the cell viability of OGD/R group, OGD/R + LY294002 group, and OGD/R + OE + LY294002 group decreased by 44.67%, 55.93%, and 45.8%, respectively ($p < 0.05$), and the cell viability of OGD/R + OE group decreased by 18.9% ($p = 0.0548$). The CCK-8 assay results showed that compared to the control group, the cell viability of OGD/R group, OGD/R + LY294002 group, and OGD/R + OE + LY294002 group decreased by 49.9%, 63.18%, and 56.93%, respectively ($p < 0.05$), and the cell viability of OGD/R + OE group decreased by 18.71% ($p = 0.0536$). Based on this, GPX4 and FTH1 expression was reduced in the OGD/R group, OGD/R + LY294002 group, and OGD/R + OE + LY294002 group (Fig. 5C, $p < 0.05$, vs. control). In the meantime, ROS and MDA levels were detected, as the pictures suggested that FSP1 overexpression decreased ROS and MDA levels

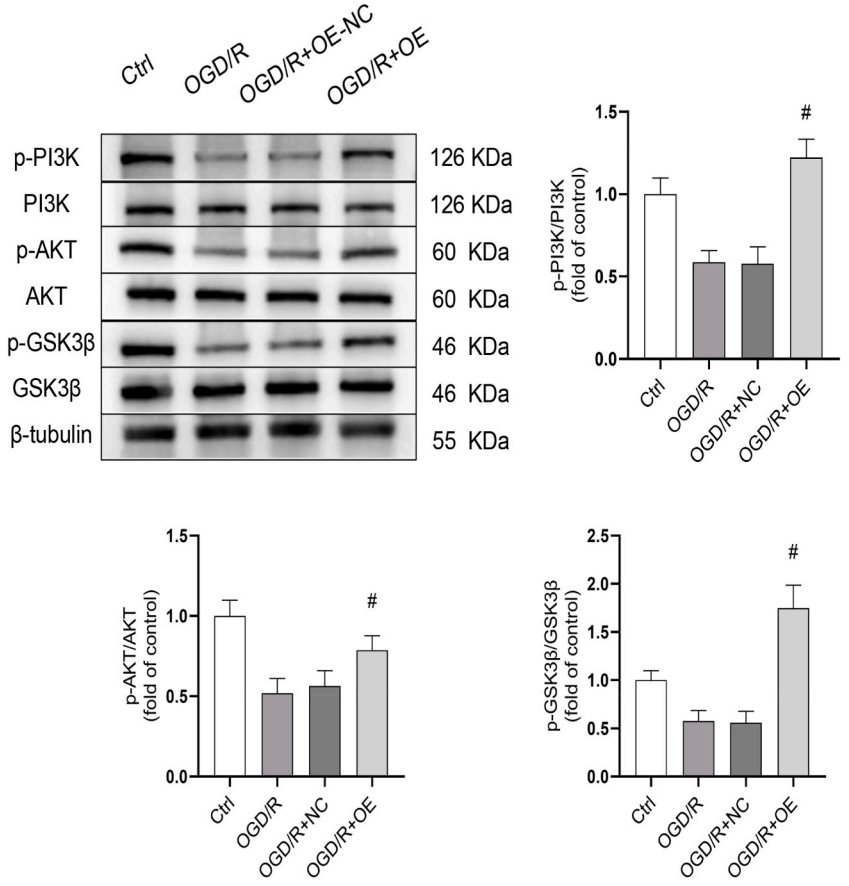

Fig. 4. The PI3K/AKT/GSK3β pathway is activated by overexpression of FSP1. Each group has representative images of Western blotting of PI3K, p-PI3K, AKT, *p*-AKT, GSK3β, and *p*-GSK3β. Overexpression of FSP1 increased p-PI3K, p-AKT, and p-GSK3β levels compared to the OGD/R group and OGD/R + OE-NC group. Values are means ± SD of data, n = 3, $^{\#}p < 0.05$ vs. OGD/R group and OGD/R + OE-NC group.

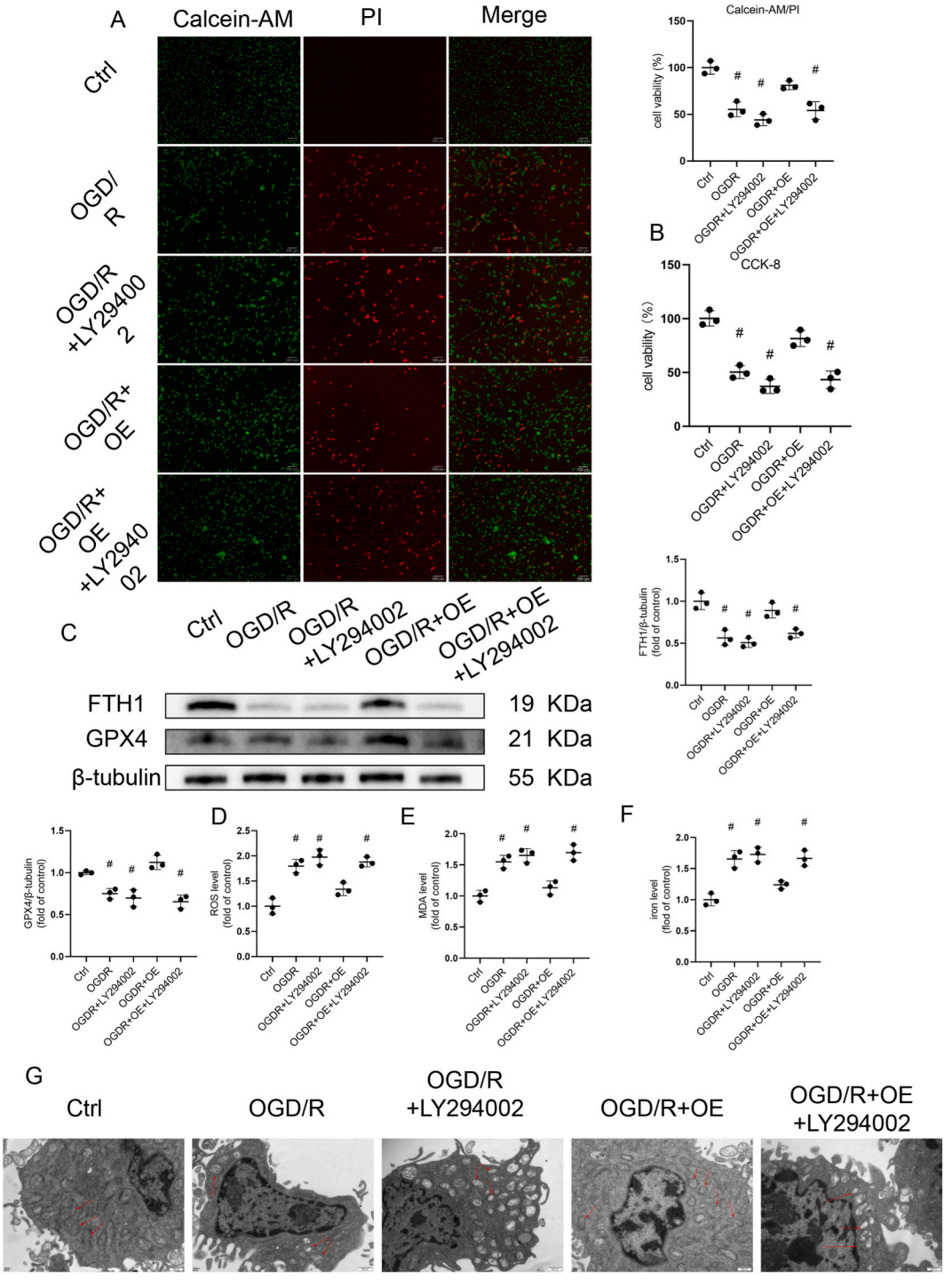

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Fig. 5. Positive effects of FSP1 overexpression are blocked by LY294002. (A) Typical pictures of Calcein-AM/PI staining shown that OGD/R, OGD/R + LY294002, and OGD/R + OE + LY294002 increased cell death compared to the control group or OGD/R + OE group. The scale bar is 100 μm. (B) CCK-8 assay showed that OGD/R, OGD/R + LY294002, and OGD/R + OE + LY294002 increased cell death compared to the control group or OGD/R + OE group. (C) Representative images of Western blotting of FTH1 and GPX4 in each group. GPX4 and FTH1 expression was reduced in the OGD/R group, OGD/R + LY294002 group, and OGD/R + OE + LY294002 group. (D) ROS assay showed that compared with the control group, ROS levels increased in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002, and ROS levels rose in group OGD/R + OE. (E) Lipid Peroxidation MDA Assay showed that compared with the control group, MDA levels increased in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002, and MDA levels rose in group OGD/R + OE. (F) Compared with the control group, iron levels increased in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002, and iron level rose in group OGD/R + OE. (G) Typical pictures of mitochondrial morphology show that Overexpression of FSP1 attenuates mitochondrial morphological changes compared to OGD/R, OGD/R + LY294002, and OGD/R + OE + LY294002. The scale bar is 500 nm. Values are means ± SD of data, n = 3, $^{\#}p < 0.05$ vs. control.

(Fig. 5D and E). Compared with the control group, ROS levels increased to 179.7%, 197.7% and 187.9% in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002 ($p < 0.05$, vs. control), respectively, and ROS levels rose to 133.8% in group OGD/R + OE ($p = 0.0713$, vs. control). Compared with the control group, MDA levels increased to 155%, 165.2% and 169.8% in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002 ($p < 0.05$, vs. control), respectively, and MDA levels rose to 113.3% in group OGD/R + OE ($p = 0.5884$, vs. control). We then measured the levels of intracellular iron in each group (Fig. 5F). Compared with the control group, iron levels increased to 165.4%, 172.7% and 166.5% in groups OGD/R, OGD/R + LY294002 and OGD/R + OE + LY294002 ($p < 0.05$, vs. control), respectively. However, iron level rose to 124% in group OGD/R + OE ($p = 0.1396$, vs. control). Meanwhile, overexpression FSP1 rescued mitochondrial pathological changes in ferroptosis, and this protective effect is blocked by LY294002 (Fig. 5G). If the PI3K/AKT/GSK3β pathway is blocked, it can significantly lessen the protective effect that overexpressing FSP1 has on OGD/R-induced ferroptosis in PC12 cells.

4. Discussion

Recent studies suggested that FSP1 could rescue ferroptosis [26–28] and may be one of the targets for treating ischemic stroke [29]. Therefore, FSP1 has great potential in preventing disease development, the detailed and well-defined molecular mechanism of FSP1 is still unknown. In our previous research, we explored how FSP1 might work and identified relevant outcomes. Ferroptosis is involved in I/R-induced neural injury. FSP1 is clearly expressed in neurons and varies according to stimulation level. Overexpression of FSP1 could against ferroptosis, and its working mechanisms are partly related to the activation of the PI3K/AKT/GSK3β pathway.

Ferroptosis is a form of PCD found in tumors, immunity, and broader biological contexts [30]. Over the course of the last decade, an increasing number of academics have focused their attention on the connection between ferroptosis and ischemic stroke [7,31,32]. GPX4, as a significant target in regulating ferroptosis, plays a role in the clearance of lipid peroxides [33,34]. Selenium supplementation promotes the expression of GPX4, which can protect neurons in treating stroke [35]. Ferritin, composed of FTH1 and ferritin light, is a complex that stores free iron and exerts antioxidant effects [36]. FTH1 can be specifically recognized by nuclear receptor coactivator 4 combined into a complex, leading to iron metabolism disorders and thus promoting ferroptosis [37]. Therefore, GPX4 and FTH1 are essential criteria for evaluating ferroptosis. Our study observed decreased GPX4 and expression after OGD/R and Erastin-induced injury. Detection of lipid peroxidation is also a means to verify whether ferroptosis occurs, including using a C11-BODIPY fluorescent probe and detecting MDA [30,38]. Oxidative damage caused by ROS and MDA has been implicated in a variety of diseases and pathological conditions, including cancer, cardiovascular disease, neurodegenerative diseases, and aging. Recent studies have shown that in some cases, it is possible to trigger cell death by inducing redox imbalance in tumor cells. One method is to increase of lipid peroxidation levels with capsaicin treatment inhibited cell proliferation by enhancing ferroptosis in glioblastoma cells [39]. Previous research suggested that OGD/R leads to increased lipid peroxidation, ROS, and MDA levels [29,32,40]. As expected, our results showed the same trend. An abnormal increase in intracellular iron ions may lead to cellular and organ damage and pathological changes. Excess intracellular iron ions may cause oxidative stress and inflammation, leading to lipid peroxidation of cell membranes and oxidative damage to DNA, resulting in cell death or irreversible damage, known as “ferroptosis” [41,42]. Our experimental results have shown that under the stimulation of OGD/R in cells, the level of intracellular iron ions increases, and overexpression of FSP1 can effectively reduce the accumulation of iron ions. Mitochondrial morphogenesis shrinks, and the reduction of mitochondrial cristae is a morphological feature specific to ferroptosis [8,43]. After OGD/R and erastin-induced injury, cells were observed under TEM with shrinking mitochondria and reduced or disappearing cristae. The above results powerfully demonstrate that ferroptosis does occur during ischemia-reperfusion injury in ischemic stroke.

FSP1 could regenerate reduced CoQ10 to scavenge lipid peroxidation intermediates [14,15]. As a vital protein against ferroptosis, FSP1 has been studied in several aspects [44–46]. However, the research on FSP1 in ischemia-reperfusion injury is just beginning. First, we observed the expression changes of FSP1. When cells were subjected to OGD/R, the expression of FSP1 first reduced, and then gradually increased during the course of the treatment. This result suggested that FSP1 may be involved in OGD/R-induced ferroptosis. Using lentiviral transfection technology, we overexpressed and interfered with the expression of FSP1. Experimental data showed that overexpression of FSP1 increased cell survival rate and decreased ROS and MDA levels. Elevated GPX4 and FTH1 levels and the mitochondrial morphology results verified that ferroptosis was alleviated. These results demonstrated that overexpression of FSP1 against ferroptosis rescues OGD/R-induced neuronal damage.

Recent studies about the PI3K/AKT/GSK3β pathway in ischemic stroke have been previously discussed [47,48]. More recent studies suggested that FSP1 was identified as oxidoreductase for CoQ10 and then reduced phospholipid peroxidation [14,49]. CoQ10,

an important molecule involved in regulating ferroptosis via FSP1, also reduces ROS production through the PI3K/AKT pathway [20]. Increased phosphorylation of AKT promotes the phosphorylation of GSK3β to gain a neuroprotective effect [50]. As a downstream molecule of FSP1, CoQ10 can excite the PI3K/AKT pathway to reduce ROS levels [20,51]. Therefore, it is reasonable to speculate that the neuroprotective effect of FSP1 against ferroptosis may be related to the PI3K/AKT/GSK3β pathway. To further investigate the potential mechanisms of FSP1, we detected the expression changes of PI3K, p-PI3K, AKT, p-AKT, p-GSK3β, and GSK3β. OGD/R resulted in a decrease in p-PI3K, p-AKT, and p-GSK3β, but FSP1-overexpression counteracted this trend. Next, we pretreated cells with an inhibitor of PI3K to block the PI3K/AKT/GSK3β pathway. The results showed that LY294002 inhibited FSP1-overexpression stimulated p-PI3K, p-AKT, and p-GSK3β. Meanwhile, LY294002 led to a decrease in cell viability. The PI3K inhibitor increased the levels of ROS and MDA. These data suggested that FSP1 improved OGDR-induced injury by activating the PI3K/AKT/GSK3β signaling pathway.

Future research should examine if FSP1 can directly upregulate PI3K. Due to the limited experimental period, in vivo experiments were not performed, which will be required for subsequent studies.

In summary, our findings in vitro demonstrated that ferroptosis does play an essential role in ischemia-reperfusion-induced neuronal injury. The work is the first to demonstrate that FSP1 acts as a neuroprotective factor in OGD/R by triggering the PI3K/AKT/GSK3β signaling pathway. The results prove that resistance to ferroptosis is one of the ways to save cerebral ischemia-reperfusion injury and provide a new research idea for the treatment of ischemic stroke. In this study, the effect of FSP1 overexpression on ferroptosis signaling was only studied in PC12 cells. One major limitation of this study is the lack of analysis on the impact of FSP1 overexpression on primary neural cells and *in vivo* models. Another limitation is that our study only provided preliminary insights into the effects of FSP1 overexpression on ferroptosis and related molecular mechanisms, with a lack of investigation into its long-term effects. Therefore, further research is needed to explore the impact of FSP1 on other ferroptosis biomarkers in ischemic stroke and investigate its long-term effects on iron metabolism and related disorders. Hence, more in-depth exploration of FSP1's other functions and mechanisms of action is required in future research to comprehensively understand its role in ischemic stroke.

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Ethical statements

The experimental protocols were approved by the Laboratory Animal Welfare and Ethics Committee of the 904th Hospital of PLA (20220215).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18449.

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