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Mesenchymal stem cells enchanced by salidroside to inhibit ferroptosis and improve premature ovarian insufficiency via Keap1/Nrf2/GPX4 signaling

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ABSTRACT

Background: Regenerative medicine researches have shown that mesenchymal stem cells (MSCs) may be an effective treatment method for premature ovarian insufficiency (POI). However, the efficacy of MSCs is still limited.

Purpose: This study aims to explain whether salidroside and MSCs combination is a therapeutic strategy to POI and to explore salidroside-enhanced MSCs inhibiting ferroptosis via Keap1/Nrf2/GPX4 signaling.

Methods: The effect of salidroside and MSCs on ovarian granular cells (GCs) was analyzed. After treatment, hormone levels and -fertility of rats were measured. Lipid peroxidation levels, iron deposition and mitochondrial morphology were detected. The genes and proteins of Keap1/Nrf2/GPX4 signaling were examined.

Results: Salidroside and MSCs were found to inhibit cell death of GCs by reducing peroxidation and intracellular ferrous. Salidroside promotes the proliferation of MSCs and supports cell survival in ovary. Salidroside combined with MSCs therapy restored ovarian function, which was better than MSCs monotherapy. Salidroside-enhanced MSCs to inhibit ferroptosis. The results showed activation of the Keap1/Nrf2/GPX4 signaling and an increase in anti-ferroptosis molecule.

Conclusions: Salidroside-enhanced MSCs as a ferroptosis inhibitor and provide new therapeutic strategies for POI. The possible mechanisms of MSCs were related to maintaining redox homeostasis via a Keap1/Nrf2/GPX4 signaling.

KEYWORDS
Salidroside; mesenchymal stem cells; premature ovarian insufficiency; ferroptosis; Keap1/Nrf2/GPX4 signaling; redox homeostasis; ovarian function; fertility

Introduction

Premature ovarian insufficiency (POI), a syndrome of ovarian hypofunction in younger women usually before the age of 40, is characterized by infertility, abnormal menstruation (amenorrhea, rare menstruation or frequent menstruation), elevated gonadotropins and decreased estrogen levels [1,2]. POI affects approximately 1∼3% of women in the general population [3,4]. However, the etiology and pathogenesis underlying POI remains unclear. Chemotherapy and radiotherapy are considered the significant causes of POI, especially the increase in young cancer patients, which caused ovarian injury [5,6]. Recently, some studies have speculated that POI was linked to ferroptosis. Ferroptosis is a form of cell death depending on iron accumulation and lipid peroxidation. Many studies have shown that oxidative stress induces reactive oxygen species (ROS) and other oxidative stress products accumulation, disorders in metabolism and signaling pathway, and damage the original follicle, resulting in reduced follicle growth [7–9]. It is reported that fluoride or cisplatin induced ovarian granulosa cell death with typical ferroptosis features, such as mitochondrial morphological abnormalities and dysfunction, resulting in impaired follicular development [10,11]. And POI patients showed mitochondrial mutations and increased ROS levels [7,12]. Iron overload-induced ferroptosis may inhibit oocyte maturation and follicle development [13,14]. In addition, Wang et al. [15] found that the deficiency of transcription factor BNC1 was found to promote POI by inducing oocyte ferroptosis. Therefore, it is speculated that the occurrence of POI may be related to ferroptosis.

Currently, there is no satisfactory treatment strategy for POI. Research in regenerative medicine has indicated that mesenchymal stem cells (MSCs) may be an effective treatment method for POI [16–18]. Additionally, other studies have reported that MSCs or its exosome can repair oxidative stress-induced damage through the Nrf2 defense system, remove ROS and inhibit ferroptosis in mice with acute myocardial infarction [19–21]. However, the MSCs therapy still had limitation. MSCs injected intravenously tend to localize in tissues such as liver and lung, resulting in a reduced number of cells returning to the target tissues. Furthermore, the pathological microenvironment (inflammation, hypoxia, ischemia, etc.) significantly impacts the survival of orthotopically transplanted MSCs, leading to increased cell apoptosis and restricting their therapeutic efficacy. Therefore, enhancing the survival of MSCs is crucial for the success of MSC-based therapies.

Salidroside is the primary active component of Rhodiola rosea with many pharmacological effects, such as anti-aging, anti-oxidation, scavenging free radical and immune

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regulation [22,23]. Zhou et al. [24] demonstrated that salidroside can promote the survival and migration potential of MSCs. Agnes et al. [25] reported that salidroside decreases intracellular ROS production induced by hyperglycemia, enhances the antioxidant capacity of MSCs and improves their paracrine function. It has been found that salidroside exhibit a wide range of pharmacological effects due to its ability to scavenge superoxide anions and hydroxyl radicals in chemical reactions [26,27]. However, whether salidroside can promote the survival and function of MSCs in POI are still unknown. In this work, we investigated the effects of salidroside on the therapeutic efficacy of MSCs-based therapy in cyclophosphamide-induced POI in rats. Our findings indicate that salidroside significantly reduced the lipid peroxidation and the apoptosis rate of MSCs. We have shown that salidroside increases the therapeutic effect of MSCs on POI. Furthermore, salidroside enchance MSCs inhibiting ferroptosis in rats through Keap1/Nrf2 signaling pathway.

Collectively, our results provide insight into a novel and potential MSC-based treatment strategy for POI.

Materials and Methods

Isolation, culture and characterization of MSCs

Human umbilical samples were donated by Shenzhen Wingor Biotechnology Co., ltd. Briefly, human umbilical cord samples were collected from fully informed healthy human volunteer donors after obtaining written informed consents were obtained from them. The umbilical cord was washed with PBS to remove the arteries and veins, then cut into small pieces of 0.5–1 mm^3 and placed at the bottom of a tissue culture dish with a small amount of culture medium. The culture dishes were then incubated at 37°C with 95% humidity and 5% CO_2. The culture medium was changed one week later, and passaging was performed 14 days after the initial setup. Cells at passages 3–6 were utilized for the subsequent experiments. According to a previously published method [28], the expression of surface marker on MSCs was detected by flow cytometry using the following antibodies: anti-human CD45 (Biolegend), anti-human CD34 (Biolegend), anti-human CD73 (Biolegend), anti-human CD90 (Biolegend), anti-human CD105 (Biolegend), anti-human CD11b (Biolegend) and anti-HLA-DR (Biolegend). Gating of the single-line cells was performed based on a combination of forward scatter (FSC) and side scatter (SSC), followed by the isolation of live cells from this population. Subsequently, cells that exhibited positive expressed were gated. To assess the multipotent differentiation of MSCs, the cells were cultured in a specific medium (all sourced from Cyagen Biosciences Inc., China) for 21 days to induce osteogenesis and adipogenesis. The osteogenic and adipogenic capacities were evaluated using Alizarin Red S and Oil Red O staining, respectively, and were observed under an inverted microscope (Olympus Corporation, Japan).

At the same time, the conditioned medium (CM) from MSCs (2×10^6 cells), and the conditioned medium from MSCs (2×10^6 cells) pretreated by salidroside (100 μg/ml), referred to as SCM, were collected and centrifuged at $1500 \times g$ for 5 min to remove cell debris. Subsequently, the medium was filtered through a 0.22 μm filter. A 3 kDa cut-off centrifugal filter unit (Millipore) was then employed to concentrate the medium 25-fold by centrifugation at $5000 \times g$ for 40–50 min. Finally, the concentrated medium was filtered and utilized in vitro therapy of rat GCs. For the in vitro cell experiments, CM or SCM was mixed with fresh DMEM/F12 complete medium in a 1:1 ratio.

Isolation, culture and characterization of primary GCs

Female Sprague–Dawley (SD) rats, aged 3–4 weeks, were acquired from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All procedures conducted in this study were approved by Southern Medical University Experimental Animal Ethics Committee. Rats were injected subcutaneously with pregnant mare serum gonadotropin (ProSpecbio, USA) at a dose of 60 IU per rat. After 48 h, the rats were sacrificed and the ovaries were harvested. The surface fat was removed, and the follicles were punctured with a syringe and adequately ground to release ovarian granulosa cells (GCs) into the medium. The GCs were washed with PBS and the medium was added; it was subsequently changed 48 h later.

Next, we sought to determine the immunophenotypes of GCs using immunofluorescent labeling technique with an FSHR antibody (Affinity Bioscience, USA). Briefly, GCs exhibiting an adhesion rate of 70% were washed with PBS and fixed with 4% paraformaldehyde. Following this, GCs were treated with 0 .1%Triton X-100 for 20 min at room temperature. After blocking with 5% skim milk for 1 h, the GCs were incubated with the FSHR antibody at 4°C overnight. Subsequently, the cells were incubated with a secondary antibody conjugated with Alexa Fluor® 488 (Boster Biological Technology, China) for 1 h at room temperature. Finally, the cells were counterstained with DAPI (Beyotime Biotechnology, China) and examined under a fluorescence microscope (Leica, Germany).

Cell counting Kit-8 (CCK-8) viability assay

For the treatment of MSCs with salidroside, MSCs were inoculated into 96-well plates at a density of 0.2×10^4 cells per well. After cell adhesion, varying doses of salidroside (Macklin, China) were added to the MSCs culture for 24∼96 h. Cell viability was measured using the CCK-8 kit (Dojing, Janpan). In addition, 4-HC (8 μg/ml) was added to induce apoptosis of MSCs, as a positive control. And cells were treated with salidroside (100 μg/ml) for 48 h. We also set up the same administration group and collected MSCs for subsequent experiments.

GCs were inoculated into 96-well plates at a density of 0.8×10^4 cells per well. Following cell adhesion, 4-HC (1 μg/ml) was added to establish a cell injury model, serving as a positive control. Cells were treated with salidroside (100 μg/ml), CM and SCM for 48 h. The effect of salidroside and CM on 4-HC-induced GCs was assessed using CCK8 kit. Similarly, we employed H_2O_2 (0.25 μM) and Erastin (10 μM) to induce cell injury, with cells treated similarly with salidroside, CM or SCM for 48 h. The impacts of salidroside and CM on cells damaged by H_2O_2 and Erastin (TargetMol, China) were analyzed. Additionally, we established a corresponding administration group and collected GCs for subsequent experiments.

Mitochondrial membrane potential assay AND Iron measurements of GCs

GCs were inoculated into 96-well plates with 0.8×10^4 cells/well. 4-HC was utilized to induce cell injury, and salidroside

or CM was added to cells for 48 h as above. The mitochondrial membrane potential of GCs from each administration group was quantified using a JC-1 Assay Kit (Beyotime Biotechnology, China). Cells were incubated with JC-1 dyeing solution at 37°C for 20 min. Then cells were washed twice with precooled JC-1 buffer solution and visualized under an inverted fluorescence microscope (Leica, Germany).

GCs were seeded in the dish, when approximately at 80% confluence; the treatment was carried out as above for 48 h. The ferrous iron of GCs from each administration group was detected using a Cell Ferrous Iron Colorimetric Assay Kit (Elabscience, China). Briefly, the supernatant was collected after cells were lyzed and was mixed with an acidic buffer incubating at 37°C for 10 min. Then the cells were measured at 593 nm with a fluorescence microplate.

Model of CTX-induced POI AND Experimental design

Adult SD rats (8 weeks old, female) were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All procedures were approved by Southern Medical University Experimental Animal Ethics Committee. These rats were randomly divided into control (Blank), POI, salidroside treatment (Sal), MSCs treatment (MSCs) and salidroside combined with MSCs treatment (Sal +MSCs) groups. To establish the POI model, the rats from the POI, Sal, MSCs and Sal + MSCs groups received cyclophosphamide (CTX, Sigma, USA) at a dose of 50 mg/kg by intraperitoneal injection on the first day and then a daily dose of 8 mg/kg for the next 14 consecutive days. Rats from the Blank group were given the same amount of PBS.

Within one week after chemotherapy, the rats were scheduled for surgery and the following corresponding treatments: POI group injected with 50 µL PBS per ovary, Sal group administered with salidroside 1 mg/50 µL per ovary, MSCs group administered with 2×10^6 MSCs/50 µL per ovary, and Sal + MSCs group injected with salidroside (1 mg) and MSCs (2×10^6)/ 50 µL per ovary. Briefly, rats were anesthetized, skinned and disinfected. Cut the skin, muscle and posterior peritoneum at 0.5~1 cm from the highest point of the rat's back. Finding the ovaries which covered with white cellulite, 50 µL of corresponding components were injected into the ovarian sac using a microsyringe.

At 4th~ 5th week after treatment, 6 rats in each group were randomly selected and anaesthetized and then the organs and blood were collected. Organs were fixed or immediately frozen in liquid nitrogen and stored at −80°C for further processing. In addition, the other rats were allowed to mate with male rats at 4 weeks after surgery.

Mating trial

To anaylsis the fertility of rats, 6 rats in each group were randomly selected for combined cage with male rats at a ratio of 2 females: 1 male after 4 weeks of surgery. The number of pregnant rats and viable births per litter were recorded in each group.

Serum preparation and hormone assays

At 4th week after surgery, blood samples were collected from the orbital venous plexus while the rats were in diestrus. After standing at room temperature for at least 1 hour, blood samples were centrifuged at 3000 rpm for 10 min to collect serum. Serum levels of follicle-stimulating hormone (FSH), anti-muller's hormone (AMH) and estradiol (E2) were detected using the ELISA kit (purchased from Jiangsu Meimian industrial Co., Ltd, China) according to the manufacturer's instructions.

Histology and follicle counting

At 4th week after surgery, six rats randomly selected from each group were sacrificed. The left ovaries were collected and fixed with 4% paraformaldehyde for 48 h and then processed for histology. The ovaries were dehydrated, embedded in paraffin and serially sectioned at a thickness of 4 µm. After Hematoxylin and eosin (H&E) staining, observed and imaged the sections by an optical microscope (Olympus Corporation). As previously described [17], follicles counting was performed by two independent experimenters without knowing the groups, recording as antral follicle (including secondary and mature follicles), total follicle count and atretic follicles.

Measurement of SOD and MDA level

To evaluate lipid peroxidation condition of ovaries, serum and GCs, the malondialdehyde (MDA) levels and the activity of superoxide dismutase (SOD) were detected using the commercially available kits (Elabscience, China). At the same time, GCs was treated with the SOD inhibitor LCS-1(Selleck.cn, China) at IC50 0.2 µM as a positive control. Ovary tissue from each group ($n = 3$) or GCs was collected and homogenized. Blood from rats was centrifuged as above to collect the serum. GCs were seeded in the dish, when approximately at 80% confluence; the treatment was carried out as above for 48 h. Collected the tissue or cells for detection according the instructions.

Transmission electron microscopy

To analysis morphology of mitochondria in tissues, ovaries were quickly collected from the rats of Blank, POI and Sal + MSCs group, sectioned at a shape of 2 mm × 2 mm. Ovarian tissue was fixed with electron microscope fixing solution for 2~4 h. Then ovarian tissue was embedded, dehydrated and cut into ultrathin sections (60–80 nm). Sections were stained with uranyl acetate for 15 min and then stained with lead citrate for 15 min. Images were acquired using Transmission Electron Microscope (HT7700, HITACHI).

Prussian Blue Iron Stain assay

To detect non-heme ferric iron and ferrous iron in rat's ovary, the Enhanced Prussian Blue Iron Stain Kit (Solarbio, China) was used. After deparaffinization and hydration, the section of ovary tissue was stained, washed, counterstained, dehydrated and cover-slipped according to the manufacturer's protocol. At least four rats in each group were employed.

Quantitative real-time PCR (RT-qPCR) and Western blot analysis

Total RNA of ovary (three rats in each group were used) or cells was extracted using commercially available kit

(Vazyme, China). MSCs or GCs were seeded at the dish, when approximately at 80% confluence; the treatment was carried out as above for 48 h. The RNA sample was quantified by Spectrophotometer.OSE-260 (TIANGEN, China) and reverse transcribed into cDNA using the cDNA Synthesis Kit (Vazyme, China). RT-qPCR was performed with SYBR Green Master Mix (Vazyme, China). Target gene expression was evaluated using the 2-ΔΔCT method, and GAPDH was used as the internal reference. The sequences of primers used in the experiment are listed in Supplementary Table 1.

Ovary tissue from rats (three rats in each group were used) or cells were lysed in RIPA buffer with protease inhibitor cocktail (Beyotime Biotechnology, China) and quantified using a BCA kit (Beyotime Biotechnology, China). Protein samples were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). After washing and blocking with 5% skim milk for 1h, the membranes were incubated with primary antibodies at 4 °C overnight; including Keap1 monoclonal antibody (Proteintech, China), Nrf2 monoclonal antibody (Proteintech, China), Anti-FACL4 antibody (abcam, UK), Anti-GPX4 antibody (abcam, UK) and recombinant anti-beta Actin abtibody (Servicebio, China). Subsequently, incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The enhanced ECL Plus kit (Clinx, China) was used in membranes visualized in ChemiDoc MP Imaging System (Bio-Rad).

Immunohistochemistry

In order to analyze the survival of MSCs in ovary tissue of rat, we detected the Hominin-specific protein by immunohistochemistry. Three rats in Blank group, MSCs group and Sal + MSCs group were employed. After deparaffinization and hydration, the ovarian sections were covered with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity. Then sections were washing and boiling in 10 mM citric acid (pH 6.0) for 15 min. Subsequently, treated it with 3% goat normal serum (Servicebio, China) for 60 min. And sections were incubated with ARHGAP11B antibody (Thermo, USA) overnight at 4°C. After washing, the sections were incubated with the secondary antibody for 30 min at room temperature. According to the manufacturer's protocol, the DAB staining kit (Beyotime Biotechnology, China) was used, and then, the sections were counterstained with hematoxylin, dehydrated and cover-slipped.

Table 1. Primer sequences used in RT-qPCR.

Gene name	Primer sequen
Rat-Nrf2	Forward, TGTCAGCTACTCCCAGGTTG
	Reverse, AGGGCAAGCGACTGAAATGT
Rat-Keap1	Forward, TGCTCAACCGCTTGCTGTATGC
	Reverse, TCATCCGCCACTCATTCCTCTCC
Rat-NQO1	Forward, AAACGACATCACAGGGGAGC
	Reverse, GGGCACCCCAAACCAATACA
Rat-HMOX1	Forward, ACAGAAGAGGCTAAGACCGC
	Reverse, TGCAGAGGTAGTATCTTGAACC
Rat-AMH	Forward, AGCAAGGGGACAGGGACAGATG
	Reverse, TTGGCAGTTGTTGGCTTGGTAGG
Rat-BMP15	Forward, GATAAAGCCGTCAGCCAGTG
	Reverse, AAGTTGATGGCGGTAGACCA
Rat-GDF9	Forward, CAATACCGTCCGGCTCTTCA
	Reverse, AAGCAGCGGAGTTGTTCAGA
Rat-GPX4	Forward, AGCCCATTCCCGAGCCTTTC
	Reverse, CGCGGGATGCACACAAGC
Rat-ACSL4	Forward, CCATATCGCTCTGTCACGCACTTC
	Reverse, CCAGGCTGTCCTTCTTCCCAAAC
Rat-SLC7A11	Forward, CCATCATCATCGGCACCGTCATC
	Reverse, TACTCCACAGGCAGACCAGAACAC
Rat-SLC3A2	Forward, GAAGGAGGCTCTGAGTTCTTGGTTG
	Reverse, GCCTGTCCTCACTGAAGTTCTTGG
Human-Nrf2	Forward, AGTCCAGAAGCCAAACTGACAGAAG
	Reverse, GGAGAGGATGCTGCTGAAGGAATC
Human-NQO1	Forward, AAGCCGCAGACCTTGTGATATTCC
	Reverse, CATGGCAGCGTAAGTGTAAGCAAAC
Human-HMOX1	Forward, TGCCAGTGCCACCAAGTTCAAG
	Reverse, TGTTGAGCAGGAACGCAGTCTTG
Human-IDO	Forward, GACGGCGATGCGGCTGATG
	Reverse, GGGTTGCCTTTCCAGCCAGAC
Human-FGF2	Forward, AGAAGAGCGACCCTCACATCA
	Reverse, CGGTTAGCACACACTCCTTTG
Human-TNF-α	Forward, CAATGGCGTGGAGCTGAGAGATAAC
	Reverse, GACGGCGATGCGGCTGATG

Data presentation and Statistical analysis

Statistical analysis was performed with the Stata13.0 software. All data are showed as mean ± standard error. One-way analysis of variance was used to determine significant differences among groups. Two-group comparisons were analyzed by the *t* test. If the data present skewed distribution or with a heterogeneity of variance, rank sum test or *t'* test was used. $P < 0.05$ were considered statistically significant.

Results

Characterization of MSCs and GCs

In Figure 1, the isolated MSCs on the third passage showed a fibroblastic morphology. Flow cytometry analysis confirmed the expression rates of surface antigens CD73, CD90 and CD105 on MSCs were higher than 95%, and those of CD11b, CD34, CD19, CD45 and HLA-DR were lower than 5%. Moreover, MSCs can induce differentiation in adipocytes and osteoblasts (Figure 1B) with a multilineage differentiation potential.

FSHR immunocytochemical staining is a specific staining of ovarian granulosa cells (GCs). As Figure 4A showed, FSHR positive staining is located in the cytoplasm of the cells, which is green stained and the nucleus is dark blue. The positive rate of FSHR over 95% under the microscope, indicating that the isolation and culture cells were GCs.

Salidroside inhibit damage induced by 4-HC of MSCs and promotes MSCs survival in ovary.

The results showed that salidroside could promote the proliferation of MSCs after treatment for 24~96 h ($P < 0.05$, Figure 1C). Salidroside protected MSCs ($P < 0.05$, Figure 2C) and reduced apoptosis rate, which induced by 4-hydroperoxy cyclophosphamide (4-HC, as the active in vitro metabolite of CTX) ($P < 0.05$, Figure 2A, B). To determine whether salidroside could affect the expression of *Nrf2*, *NQO1*, *HMOX1*, *IDO*, *FGF2* and *TNF-α* gene in MSCs, RT-qPCR was performed. Nrf2 is released and translocated to the nucleus, where it induces the expression of antioxidant elements, such as NQO1, HMOX1 and GPX4, which has been reported to be associated with POI treatment. IDO, FGF2 and TNF-a are important paracrine factors of MSC, which may be related to the repair damage of the tissue. As shown in Figure 1D, compared to the control group, the relative mRNA expression of *Nrf2*, *NQO1*, *HMOX1*, *IDO* and *FGF2* in MSCs of MSCs + Sal group were significantly higher ($P < 0.05$). The relative mRNA expression of *TNF-α* in MSCs was reduced after

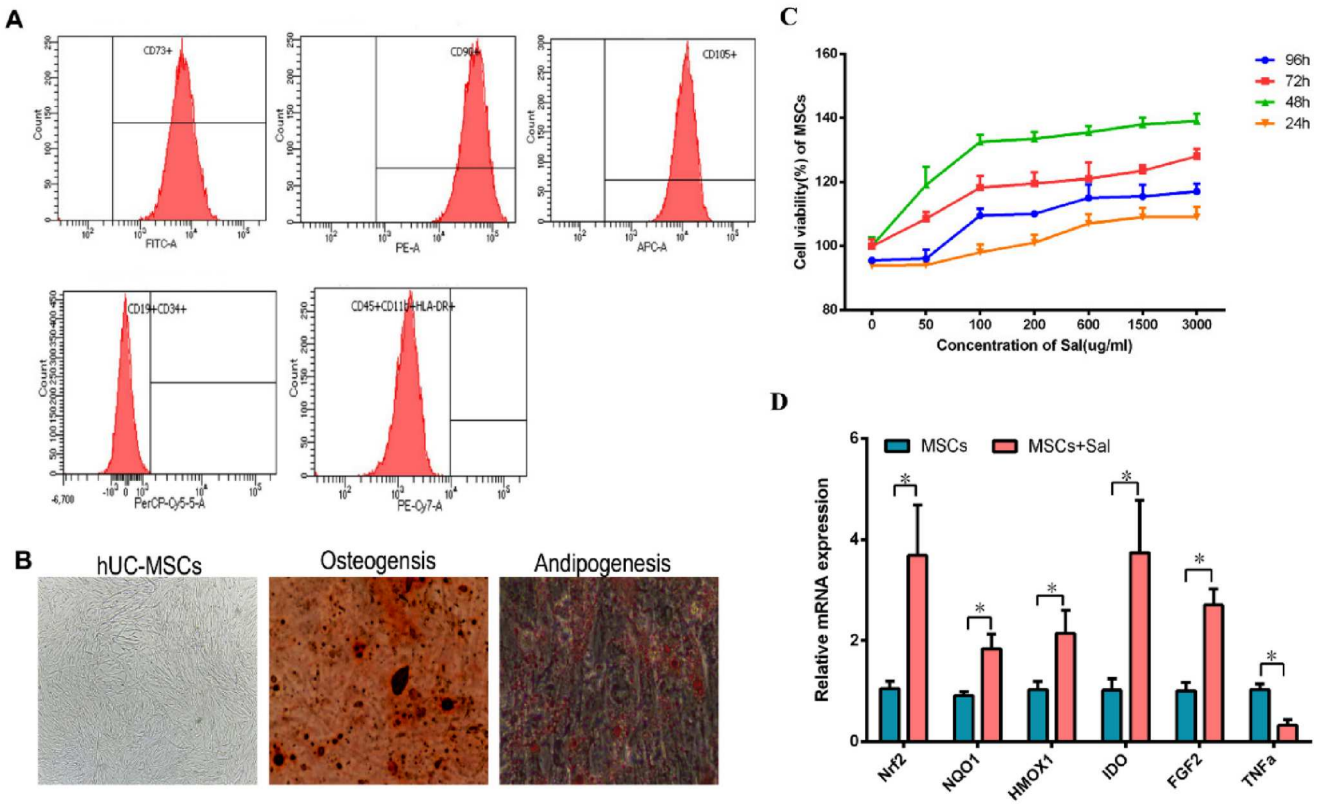

Figure 1. Identification of MSCs and the effect of salidroside on MSCs. (A) Surface antigens of MSCs by flow cytometry analysis. (B) Morphology of MSCs and its osteogenic/adipogenic differentiation potential of MSCs (100×). (C) Different doses of salidroside promote MSCs proliferation. (D) Effect of salidroside on Nrf2, NQO1, HMOX1, IDO, FGF2 and TNF-α mRNA expressions of MSCs (*$P < 0.05$ compared with the MSCs group).

treated with salidroside ($P < 0.05$). These results demonstrate that salidroside could promote MSCs from chemotherapy. It might be related to the activation of Nrf2 signaling and promote the expression of antioxidant-related genes, such as *NQO1* and *HMOX1*. As well as altered levels of *IDO*, *FGF2* and *TNF-a* mRNA, we also hypothesized that it may also promote the paracrine of MSCs.

We further evaluated whether salidroside could promote the survival of MSCs in ovarian tissue of POI rats. The results from immunohistochemistry showed that more positive expression in the Sal + MSCs group at 2nd week and 4th week after treatment, compared with MSCs group. In particularly, the positive expression was obvious in the Sal + MSCs group at 4th week, while the less MSCs in rats which only received MSCs injection (Figure 2D). These results indicate that salidroside could promote MSCs subsist in ovary of POI rats induced by chemotherapy.

Salidroside and CM inhibit ferroptosis of GCs induced by 4-HC

We explored the cell viability of different dose of 4-HC on GCs and then selected median lethal dose (1 μg/ml) to induce in vitro cell damage model. To confirm the effect of salidroside or conditioned medium (CM) from MSCs treatments on rat GCs, we first examined the activity of the cells. And we found that salidroside, CM and SCM could effectively protect the cells damaged by 4-HC (Figure 3A). Intriguingly, CM or SCM were effectively protect GCs from death induced by erastin ($P<0.05$, Figure 3A), while salidroside monotherapy has no effect on the cells. Remarkably, all administration groups could not rescue cell viability in H_2O_2-induced death (Figure 3A), indicating salidroside or CM from MSCs was a selective inhibitor of ferroptosis.

As lipid peroxidation is a character of ferroptosis, we next determined the lipid-associated products (MDA) and activity of antioxidant enzymes (SOD) of GCs, which serve key roles in maintaining the balance of oxidation and reduction. The results showed an increase of MDA and a decrease of SOD in GCs induced by 4-HC (Figure 4C). Consistently, salidroside and SCM blocked the effects of 4-HC on MDA and SOD. We also determined the influence of salidoside and CM on lipid peroxide levels in rats and the expression of acyl-CoA synthetase longchain family member 4 (ACSL4), which is an essential component for lipid peroxide accumulation [29]. The RT-qPCR results of *ACSL4* mRNA significantly up in GCs induced by 4-HC in vitro, while the expression was downregulation after treated by CM (Figure 3B). This was also confirmed in western blot as shown in Figure 3C. However, the decreased expression of *ACLS4* in salidroside monotherapy group was not as obvious as that in the CM group. The SCM treatment still to be the most effective in these results.

Next, we examined changes in mitochondrial membrane potential and Intracellular ferrous ions of GCs, which were important feature of cell ferroptosis. As shown in Figure 4B, 4-HC induced a fluorescence shift from red to green in GCs, meaning mitochondrial membrane potential decreases. After salidroside or CM treatment, the mitochondrial membrane potential of GCs increased slightly, observing more red fluorescence compared with the 4-HC-induced group. And the treatment of SCM had more red fluorescence than the other treatment groups. Furthermore, after 4-HC induced, the content of ferrous iron was obviously increased and those in all administration group showed a decreasing trend ($P<0.05$),

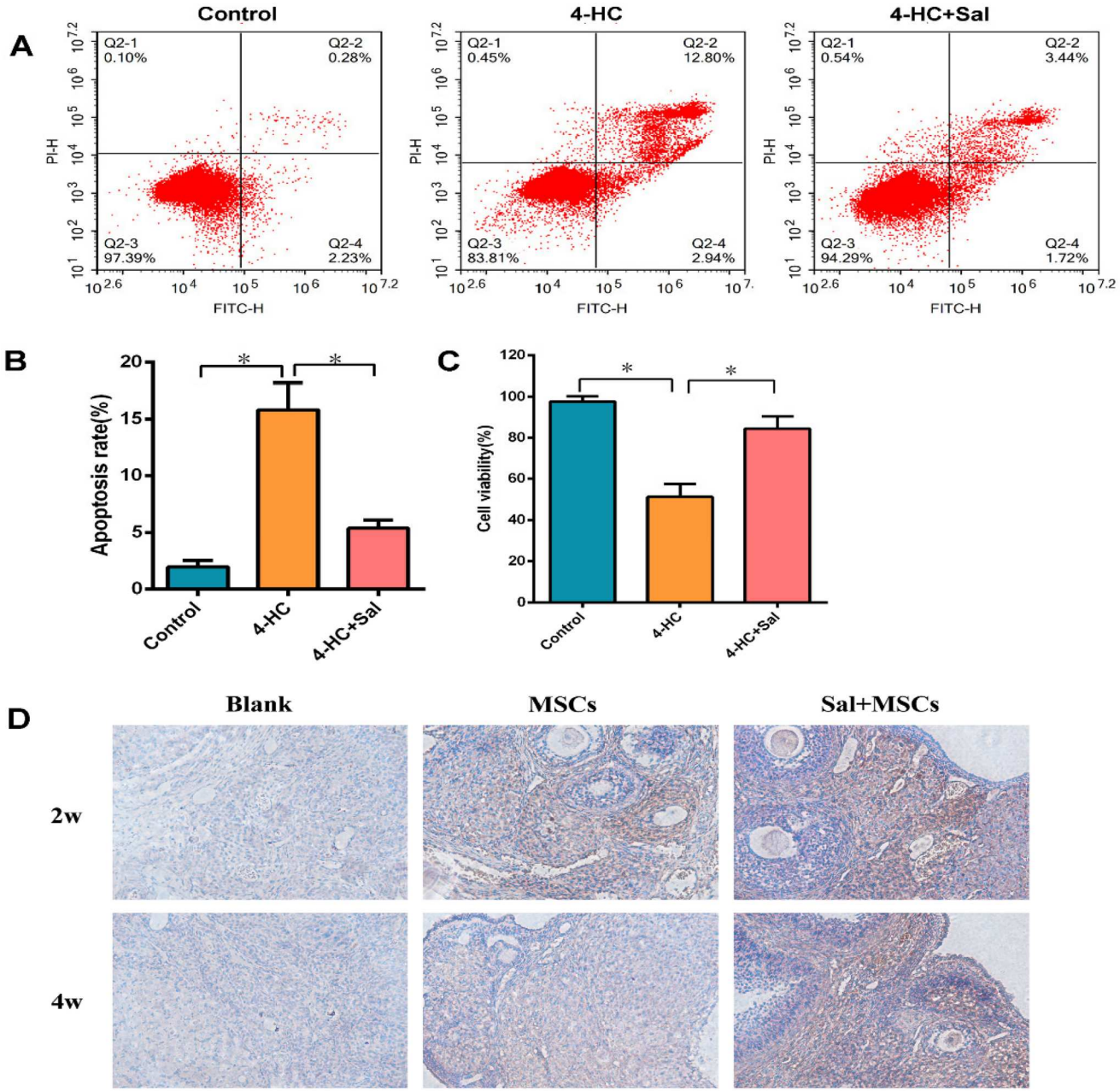

Figure 2. Effects of Salidroside on MSCs. (A) Representative apoptosis results of MSCs after administrations. (B) Apoptosis rate of MSCs after administrations (*$P <$ 0.05 compared with the 4-HC group). (C) Cell viability of MSCs after administrations (*$P < 0.05$ compared with the 4-HC group). (D) Representative IHC staining results of MSCs in ovaries at the 2nd week and 4th week after surgery (100×).

especially in the SCM group ($P<0.05$, Figure 4D), indicating that the treatment of salidroside, CM and SCM inhibited the mitochondrial membrane potential decrease and ferrous iron increase of GCs induced by 4-HC in vitro.

Together, these data support the hypothesis that GCs induced by 4-HC might exist cell ferroptosis, characterized by a decreased cell viability, altered mitochondrial membrane potential, increased lipid peroxidation and ferrous iron. Salidroside was similar to CM in regulating cell viability and oxidative stress but seems to have minor effect on mitochondrial membrane potential and lipid-related gene *ACLS4*. The effect of SCM was obviously higher than other.

MSCs enhanced by salidroside improve ovarian function and fertility of POI rats

Notably, we observed a significant increase in the number of antral follicle following treatment of MSCs and Sal + MSCs group compared with the POI group ($P < 0.05$, Figure 5A, B). In particular, the number of antral follicle in the Sal + MSCs group was more than MSCs monotherapy ($P < 0.05$). Treatment in either group reduced the atretic follicles ($P < 0.05$, Figure 5A, B). However, no significant difference in atretic follicles was observed between treatment groups.

In order to investigate the effects of administration on reproductive hormones, the hormonal levels of FSH, AMH and E2 were measured. Results showed that the FSH level of rats in the MSCs and Sal + MSCs groups declined ($P <$ 0.05, Figure 5E). The AMH and E2 level of rats in the MSCs and Sal + MSCs groups were higher than that in the POI group ($P < 0.05$, Figure 5C, D). Although salidroside monotherapy appears to have no change in these hormones, it combined with MSCs therapy exert optimal efficiency. RT-qPCR was conducted to gain further insight into the effect of salidroside and hUC-MSCs on the ovarian function. The results showed upregulation of the *AMH*, *BMP15* and *GDF9*

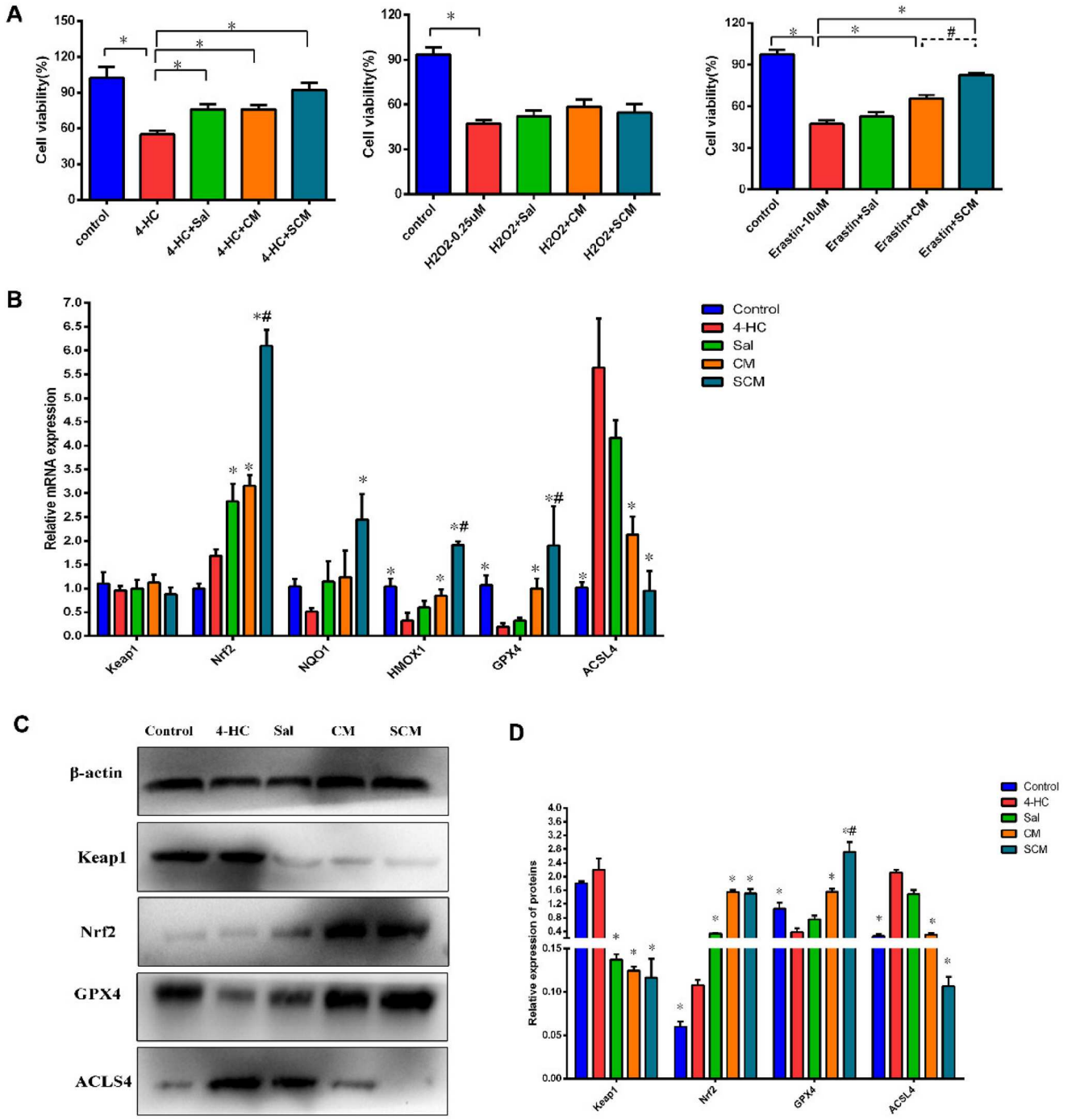

Figure 3. Effects of salidroside and conditioned medium from MSCs on 4-HC-induced Granule cells. (A) Effect of salidroside and conditioned medium on cell viability of Granule cells induced by 4-HC, H_2O_2 and Erastin, respectively (*P<0.05 compared with the 4-HC group,# P<0.05 compared with the Sal group or the CM group). (B) Quantitative analysis of the determination of Keap1, Nrf2, NQO1, HMOX1, GPX4 and ACSL4 by qPCR in Granule cells (*P < 0.05 compared with the 4-HC group,# P<0.05 compared with Sal group or CM group). (C) Detection of Keap1, Nrf2, GPX4 and ACSL4 proteins in Granule cells by western blot. (D) Statistical diagram of relative expression levels of Keap1, Nrf2, GPX4 and ACSL4 proteins (*P < 0.05 compared with the 4-HC group, # P<0.05 compared with the Sal group or the CM group)

genes in the sal + MSCs group (P < 0.05, Figure 7C). It is generally accepted that BMP15 and GDF9 are important regulatory molecules for the maintenance of ovarian function. These results showed that salidroside combined with MSCs had a better therapeutic effect on follicle recovery in POI rats.

We further assessed the fertility of POI rats. The rats received treatment of salidroside (3 out of 6 rats) and MSCs (3 out of 6 rats) became pregnant and produced live offspring (Figure 6). And the pregnancy rate of rats in Sal + MSCs group was higher (6 out of 6 rats) (Figure 6B). While the rats received salidroside monotherapy showed no difference of pups per litter compared with POI rats (3 out of 6 rats) (Figure 6B). In addition, the number of pups per litter increased significantly in the Sal + MSCs group, compared with the POI group (P<0.05, Figure 6C). These results suggest that salidroside could significantly promote MSCs restore ovarian function and promotes fertility of POI rats.

MSCs enhanced by salidroside to inhibit ferroptosis in POI rats

As ferroptosis is dependent upon ferrous deposition and lipid peroxide accumulation, we first determined the influence of salidroside and/or MSCs on lipid peroxide levels and the

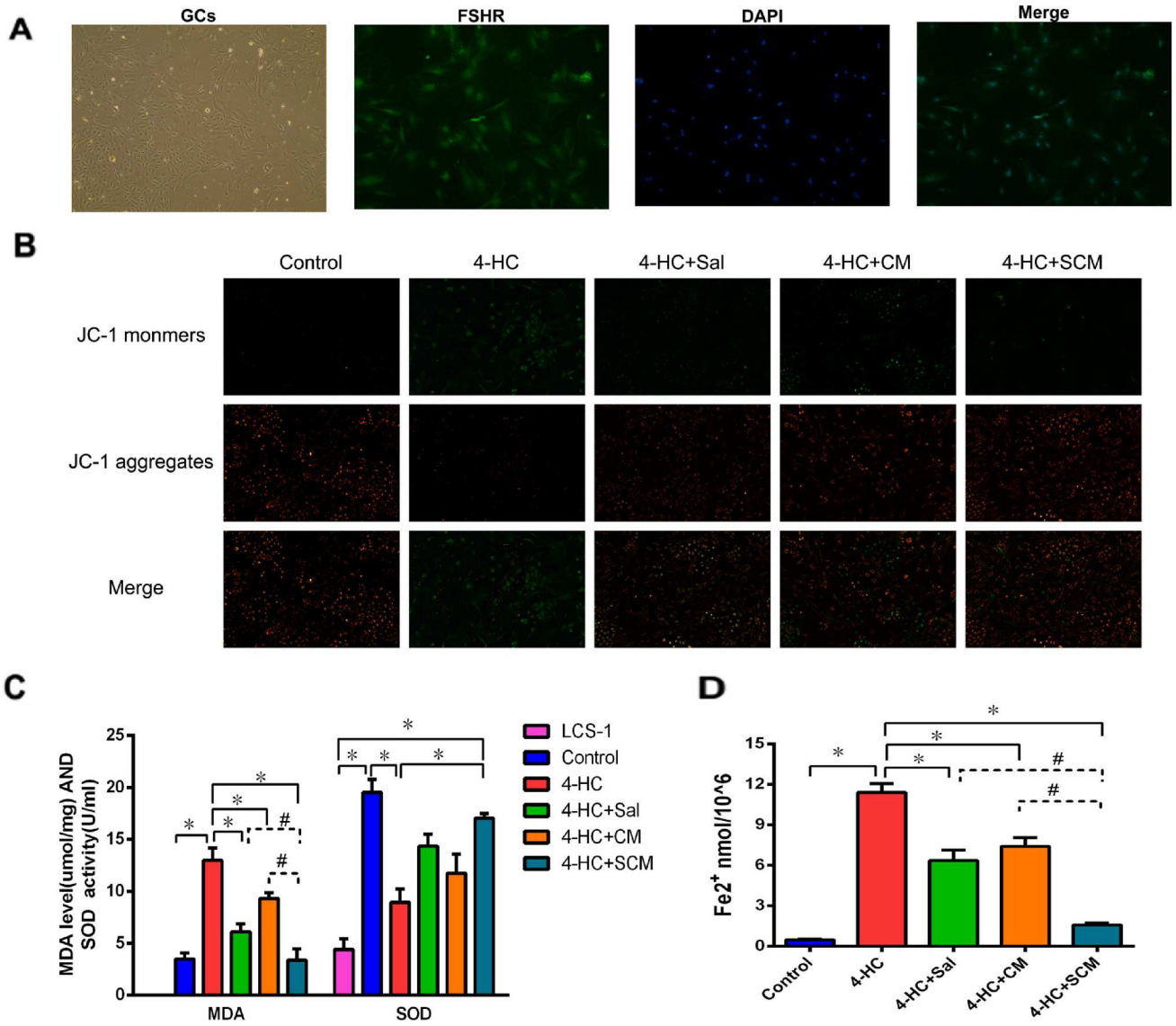

Figure 4. Identification, mitochondrial membrane potential and lipid peroxidation levels of ovarian granulosa cells. (A) Representative IF staining results of Granule cells. (B) Representative mitochondrial membrane potential detection of Granule cells in each group. (C) MDA level and SOD activity of Granule cells after administration (*$P < 0.05$ compared with the LCS-1 group or the 4-HC group, # $P<0.05$ compared with the Sal group or the CM group). (D) Ferrous ion level of Granule cells after administration (*$P < 0.05$ compared with the 4-HC group, # $P<0.05$ compared with the Sal group or the CM group).

expression of ACSL4. As shown in Figure 7A, the SOD activity of ovaries was increased in the Sal and Sal + MSCs groups ($P < 0.05$), and SOD activity of serum was increased in the Sal + MSCs groups ($P < 0.05$). The MDA level decreased in the Sal and Sal + MSCs groups either serum or ovary, particularly obvious in the Sal + MSCs group ($P < 0.05$, Figure 7B). Moreover, decreased expression levels of *ACSL4* mRNA were found in the MSCs and Sal + MSCs groups ($P < 0.05$, Figure 7C). Correspondingly, the results of Western blot showed decreased expression of ACSL4 after MSCs treatment ($P < 0.05$, Figure 7D).

Next, we explored that morphology of mitochondria and ferrous deposition in rat's ovary. The results showed that compared with normal rats, mitochondrial membrane defect and shrinkage occurred in the ovarian tissue of POI rats, while the mitochondrial structure of POI rats treated with salidroside combined with MSCs was more complete (Figure 8B). In addition, Prussian Blue Iron Stain assay showed that compared with normal rats, intense staining was observed in ovarian stroma of POI rats (Figure 8A). Iron staining decreased clearly in the administration group, especially in the Sal + MSCs group (Figure 8A). These results indicate that salidroside enhanced MSCs to inhibit ferroptosis in POI rats by alleviating the peroxidation and reducing excessive iron accumulation. These in vivo findings fit well with in vitro evidence that salidroside combined MSCs might be a promising treatment for POI by inhibiting ferroptosis.

MSCs enhanced by salidroside inhibit ferroptosis through Keap1/Nrf2/GPX4 signaling

Next, we embarked to explore the molecular mechanism that may account for the MSCs inhibition of ferroptosis. We evaluated the activation of the Keap1/Nrf2 signaling in GCs and animal ovarian tissue. Firstly, the RT-qPCR results showed that the expression of *Nrf2* mRNA in GCs increased slightly after 4-HC inducing. And expression of *Nrf2* significantly increased after salidroside, CM or SCM treatment, compared with 4-HC-induced group (Figure 3B). And expression of Nrf2 protein in Western blot assay was also consistent with this tendency ($P<0.05$, Figure 3C, D). Interestingly, the mRNA

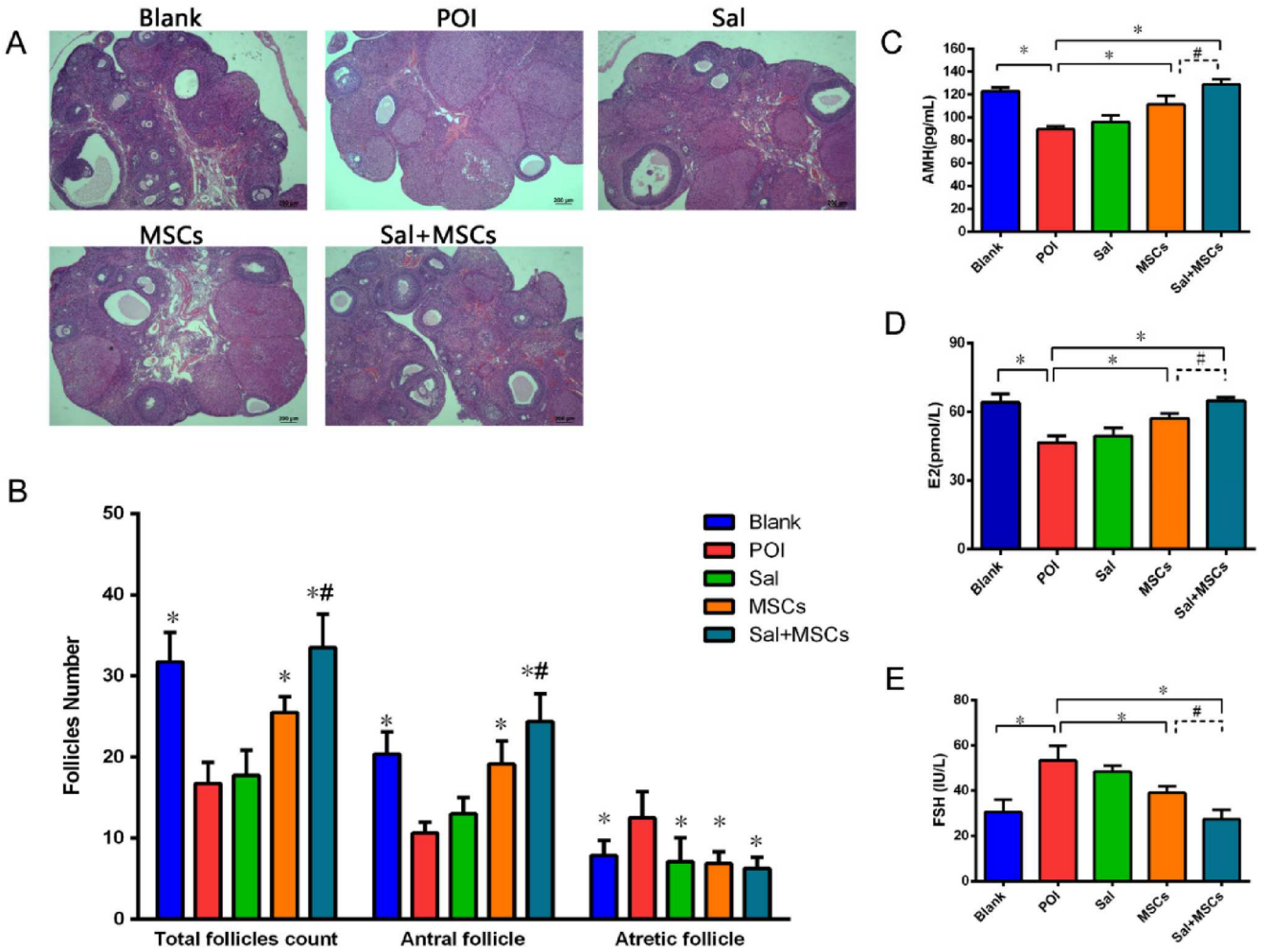

Figure 5. Effects of salidroside and MSCs on ovarian function in POI rats. (A) Representative HE staining results of ovaries in each group on the 4th week after surgery (100×). (B) Follicle counting in each group at 4th week after surgery ($n = 6$. *$P < 0.05$, compared with the POI group, # $P<0.05$ compared with the Sal group or the MSCs group). (C,D,E) FSH, E2 and AMH levels in each group at 4th week∼5th week after surgery ($n = 6$. *$P < 0.05$, compared with the POI group, # $P<0.05$ compared with the Sal group or the MSCs group).

expression of *Keap1* was not obviously different among the groups, while the results of Western blot showed that the expression of Keap1 protein was decreased in all administration groups ($P<0.05$, Figure 3C, D). We then explored the downstream genes of Keap1/Nrf2 signaling in GCs, and results revealed a dramatic decrease of *GPX4*, *NQO1* and *HMOX1* mRNA in the 4-HC-induced group, while CM or SCM rescued the defective expression ($P<0.05$, Figure 3B).

The activation of Keap1/Nrf2 was also confirmed in vivo. Results of RT-qPCR confirmed salidroside combined with MSCs could up-regulate the expression of *Nrf2* ($P<0.05$, Figure 7C). However, neither salidroside nor MSCs had no effect on expression of *Keap1*, *HMOX1* and *NQO1* in each group, but MSCs treatment significantly increased the expression of *GPX4* ($P<0.05$, Figure 7C), which is a key molecule to inhibits ferroptosis. Similar to the results in vitro, the expression of Keap1 protein was decreased, Nrf2 and GPX4 protein were increased in all administration groups ($P<0.05$, Figure 7D). In addition to iron overload and lipid peroxidation, ferroptosis is also closely related to amino acid transport, which may affect the expression of SLC7A11 and SLC3A2, two subunits of the Xc-system responsible for amino acid transport. We also found the expression of *SLC7A11* and *SLC3A2* mRNA was lower in POI rats, but upregulated by salidroside and/or MSCs ($P<0.05$, Figure 7C). These findings fit well with in vitro evidence that salidroside-enhanced MSCs inhibit ferroptosis through the activation of Keap1/Nrf2/GPX4 signaling.

Discussion

Premature ovarian insufficiency (POI), a disorder of ovarian dysfunction, seriously threatens female reproductive health. Approximately, 1,000,000 new cancer diagnoses annually worldwide, whom are adolescents and young adults aged 15–39 years [30]. Chemotherapy and radiotherapy improved the prognosis of patients but also damaged ovarian function of patients [31–33]. Protecting the ovarian function and improving the fertility of patients are being paid more and more attention. Mesenchymal stem cells (MSCs) have been described as a potential treatment for POI in both preclinical animal models and human clinical trials [34–37]. We present here salidroside-enhanced MSCs as an option to improve ovary function and preserve fertility for cyclophosphamide (CTX)-induced POI. In our work, after MSCs treatment, the number of follicles in POI rats increased significantly, accompanied by a decrease in FSH levels and an increase in E2 and AMH levels, meaning improvement in ovarian function. In addition, our study showed that MSCs treatment increased the mRNA expression of *BMP15* and *GDF9* in rats, which regulate follicle survival and oocyte maturation [38]. We further verified the effect of

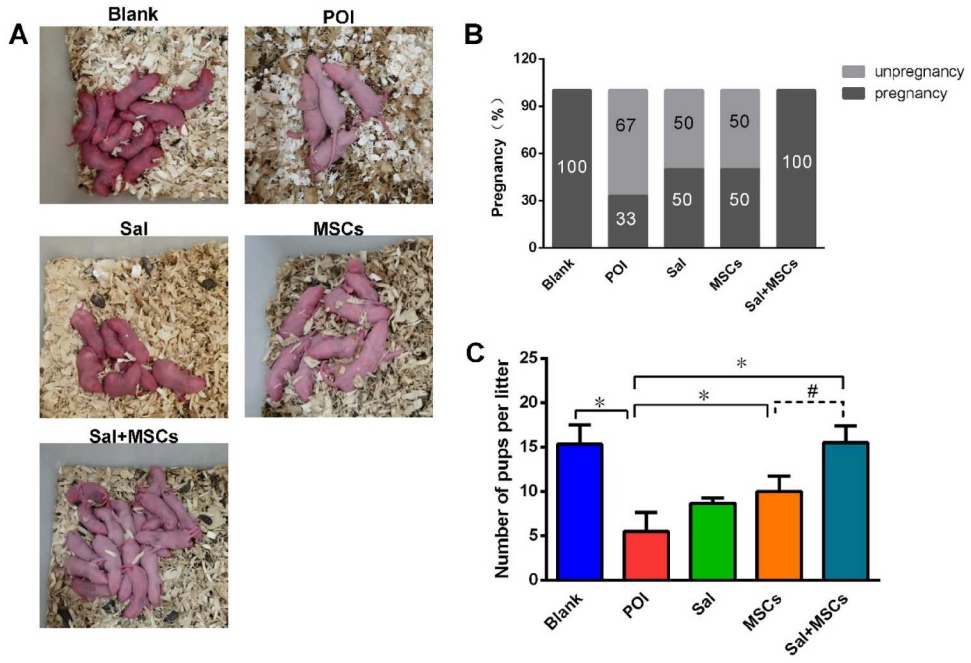

Figure 6. Effect of salidroside and MSCs on fertility in POI rats. (A) Representative pups per litter results of each group. (B) Pregnancy rate in each group after administration ($n = 6$). (C) Number of pups per litter in each group after administration ($n = 6$. $^*P < 0.05$, compared with the POI group, # $P<0.05$ compared with the Sal group or the MSCs group).

MSCs on the fertility of POI rats, and the results showed that compared with POI rats, MSCs increased pregnancy rate and pups. However, the effect of MSCs monotherapy in the treatment of POI is not fully satisfied. Some studies have reported that the survival rate of transplanted MSCs is low in vivo [39–42]. The microenvironments, such as ischemia, hypoxia and inflammation, can lead to cell death, reducing its therapeutic effectiveness [25,43]. Ling et al. [44] reported that low-intensity pulsed ultrasound pretreatment of MSCs could promote the secretion of IGF-1, HGF and VEGF factors and improve the therapeutic effect in POI rats. Chen et al. [45] found that the apoptosis rate of MSCs was significantly reduced after heat shock, and the pretreated cells had enhanced tolerance to the chemotherapy microenvironment in POI rats. Therefore, improving cell survival in target tissues is a key in MSCs therapy. Yin et al. [46] showed that salidroside had a protective effect on retinal pigment epithelial cells induced by H_2O_2. Salidroside protects cells from lipopolysaccharide-induced oxidative stress by promoting the expression of antioxidant enzymes such as SOD and reducing MDA and LDH [47]. And salidroside also promote the survival of MSCs and improve the paracrine of MSCs [24,25]. However, the effect of salidroside on POI has not been reported. Actually our results also suggest that salidroside could promote the proliferation of MSCs in vitro and reduce the apoptosis of MSCs induced by 4-HC (the active metabolite of CTX). Moreover, our study has demonstrated that compared with MSCs monotherapy, salidroside and MSCs combined injection into the ovary significantly improves the therapeutic effect, including follicle count, hormone levels and fertility. Salidroside monotherapy seems not improve ovarian function obviously, but it could reduce peroxidation, especially in ovary. Salidroside as an antioxidant reduced oxidative stress injury, including decreased MDA level and increased SOD activity in the POI rats. In addition, we analyzed the MSCs in the ovaries at 2nd week and 4th week after treatment by immunohistochemical techniques and found that salidroside could enhance the presence of MSCs. Therefore, salidroside might be beneficial for MSCs-based therapy in POI.

Ferroptosis, a form of cell death characterized by iron overload, ROS accumulation and lipid peroxidation, is accompanied by mitochondrial morphological changes, such as shrunken mitochondrial, reduced cristae and ruptured outer membrane [48,49]. Currently, there are few studies on the relationship between ferroptosis and POI. Zhang et al. [50] found that iron death may be involved in follicular atresia at an early stage, while the classical apoptosis mechanism may start in the late stage of follicular atresia. Du et al. [51] reported that cisplatin regulates the expression of ACSl4, ALOX15, SLC7A11 and GPX4, promotes ferroptosis of ovarian granulosa cells and causes ovarian hypoplasia and ovarian fibrosis in rats. Another research found that deficiency of BNC1 affects NF2-YAP-TFRC/ASCL4 signaling, causing upregulation of TFRC and ACSL4, ultimately leading to oocyte ferroptosis and follicular atresia [15]. In our study, significantly enhanced staining of iron deposition in ovary and damaged mitochondrial outer membranes in CTX-induced POI rats were observed, which were consistent with ferroptosis. These indicative ferroptosis may be one of the pathological mechanisms of POI. A lot of evidence suggests that the key molecular mechanism of ferroptosis is to regulate the balance between cellular membrane oxidative damage and antioxidant defense. Cell membrane contains polyunsaturated fatty acids, which are easily induced by ROS and catalyzed by Fe^{2+} to form toxic lipid peroxides [52,53]. In addition, overloaded intracellular iron ions increase the level of reactive oxygen species through Fenton reaction, resulting in cytotoxic effects [54]. ROS, MDA and SOD are important biomarkers of oxidative stress injury, which have been reported abnormal in animal and clinical patients of POI [55–58]. Herein, we have demonstrated that CTX-induced POI rats showed increased MDA levels and decreased SOD activity.

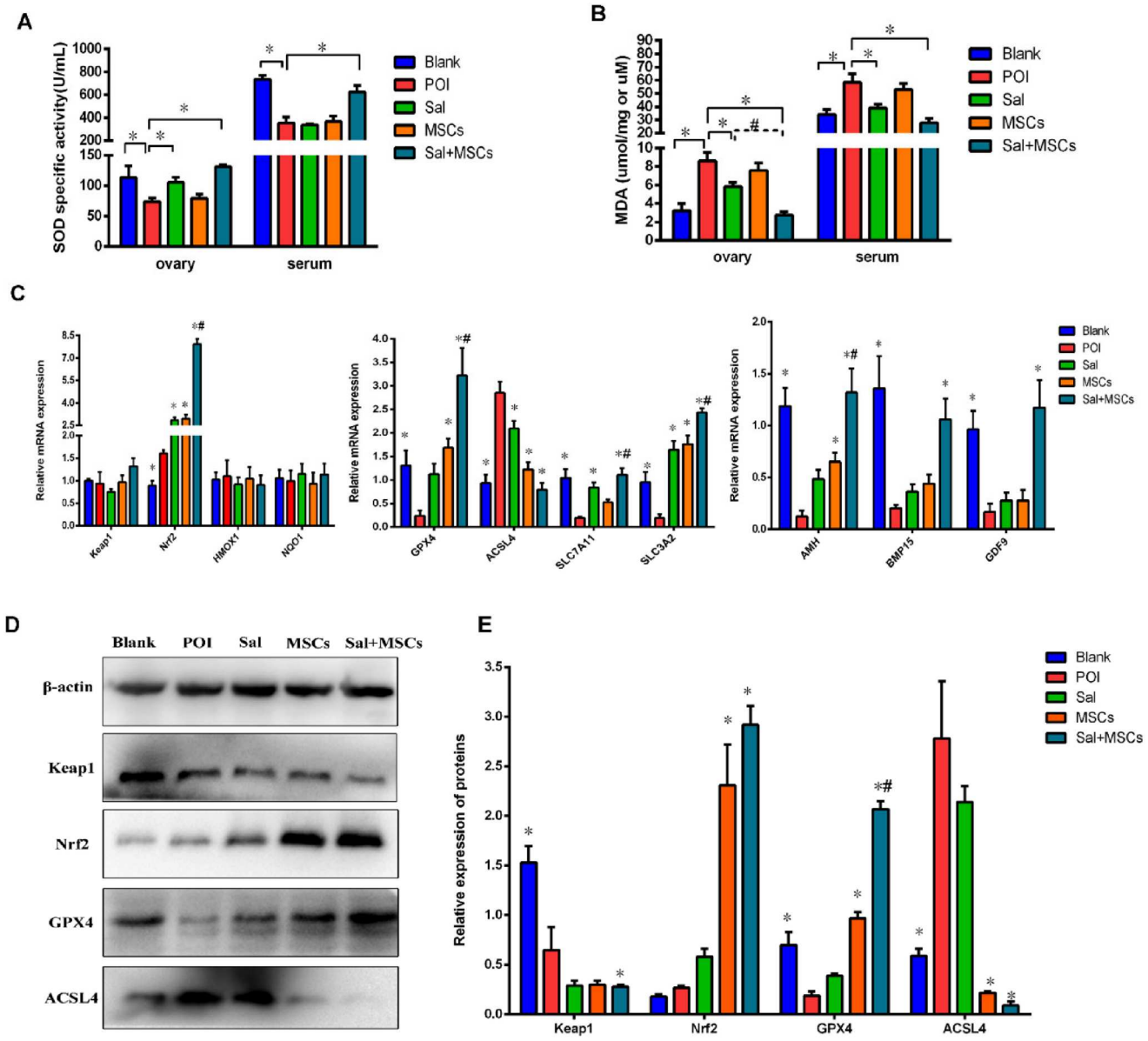

Figure 7. Lipid peroxidation and expression of related genes in Keap1/Nrf2 signaling pathway of POI rats. (A) MDA level of rats after administration (*$P < 0.05$ compared with the POI group,# $P<0.05$ compared with the Sal group or the MSCs group); (B) SOD activity of rats after administration (*$P < 0.05$ compared with the POI group,# $P<0.05$ compared with the Sal group or the MSCs group). (C) Quantitative analysis of the determination of Keap1, Nrf2 and other genes by qPCR (*$P <$ 0.05 compared with the POI group,# $P<0.05$ compared with the Sal group or the MSCs group). (D) Detection of Keap1, Nrf2, GPX4 and ACSL4 proteins of rats by western blot. (E) Statistical diagram of relative expression levels of Keap1, Nrf2, GPX4 and ACSL4 proteins (*$P < 0.05$ compared with the POI group, # $P<0.05$ compared with the Sal group or the MSCs group).

Consistently, GCs damaged by 4-HC in vitro also exhibit oxidative stress damage, elevated intracellular ferrous and decreased mitochondrial membrane potential.

Therefore, inhibiting ferroptosis by maintaining oxidative homeostasis may be an effective way to improve ovarian function of POI. Other studies have reported that MSCs can resist oxidative stress and inhibit ferroptosis [59–61]. Our study found that after MSCs treatment, the above characteristics of ferroptosis in POI rats were significantly improved. We further explored the effect of MSCs on the Keap1/Nrf2 signaling. Keap1/Nrf2 signaling pathway is an important endogenous antioxidant pathway and a key regulator of ferroptosis, regulating iron transport, antioxidant enzymes and genes related to amino acid metabolism [62,63]. GPX4, a key regulator of ferroptosis and a transcriptional target of Nrf2, converts toxic lipid hydroperoxide to non-toxic lipids to resist ferroptosis [64,65]. Another important gene promoting ferroptosis, ACSL4, is closely related to lipid metabolism of cell membranes or membrane-bound organelles. Polyunsaturated fatty acids in membrane lipids can form toxic lipid hydroperoxide with Coenzyme A (CoA) under the action of ACSL4 [66]. Our results showed that salidroside or MSCs could up-regulate Nrf2 expression and reduce Keap1 expression, and the expression of downstream gene was increased, indicating the activation of Nrf2 signaling. Although a slightly elevated Nrf2 expression was observed in the model group, we thought it may be a response to CTX or 4-HC. The elevated GPX4 and reduced ACSL4 expression were observed after MSCs treatment, which were not obvious in salidroside monotherapy. Therefore, we hypothesized that MSCs enhanced by salidroside activate Keap1/Nrf2 signaling pathway, promote Nrf2 into nuclear and up-regulate GPX4 expression, inhibiting ferroptosis. MSCs enhanced by salidroside might be a beneficial therapy for POI.

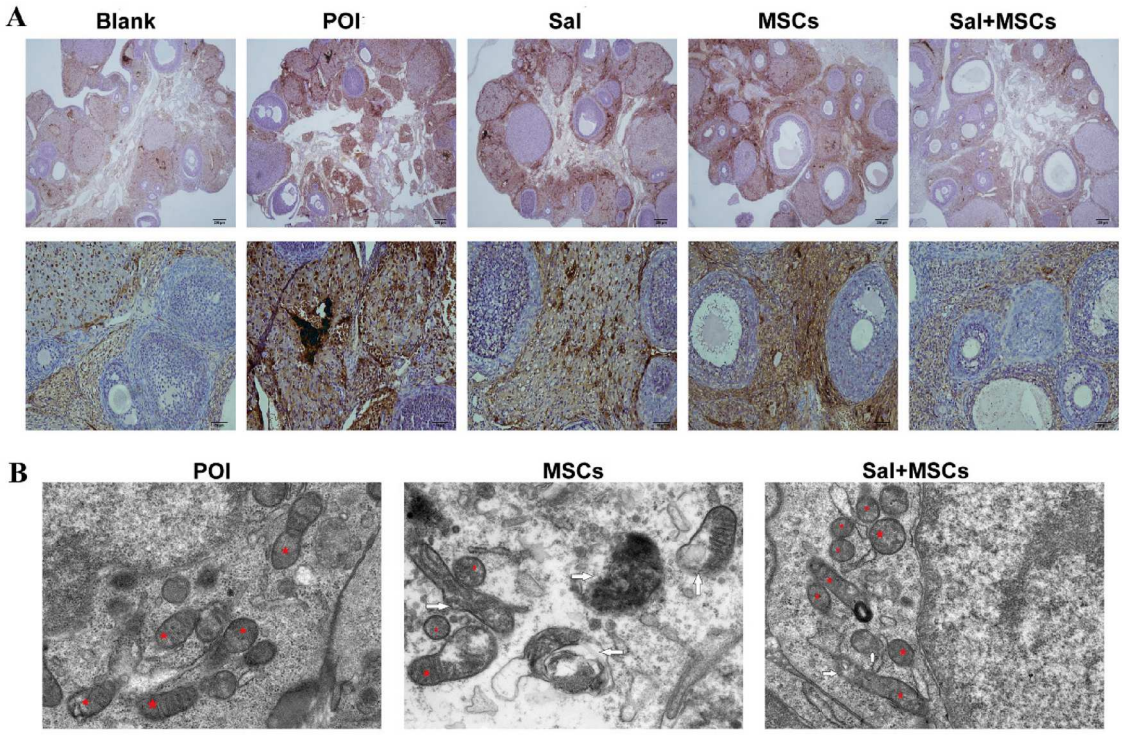

Figure 8. Iron deposition and mitochondrial morphology in ovary of each group. (A) Representative Prussian blue staining results of ovaries in each group on the 4th week after surgery (100× and 200× respectively). (B) Representative TEM results of mitochondria in each group on the 4th week after surgery.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical statement

This study has been approved by Southern Medical University Experimental Animal Ethics Committee (ethical approval number: SMIUL2022053). All the procedures complied with relevant guidelines for the care and use of laboratory animals.

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References

[1] Touraine P. Premature ovarian insufficiency: step-by-step genetics bring new insights. Fertil Steril. 2020;113(4):767–768. doi:10.1016/j.fertnstert.2019.12.032
[2] Jankowska K. Premature ovarian failure. Menopausal Rev. 2017;16(2):51–56. doi:10.5114/pm.2017.68592
[3] Shamilova NN, Marchenko LA, Dolgushina NV, et al. The role of genetic and autoimmune factors in premature ovarian failure. J Assist Reprod Genet. 2013;30(5):617–622. doi:10.1007/s10815-013-9974-4
[4] Szeliga A, Calik-Ksepka A, Maciejewska-Jeske M, et al. Autoimmune diseases in patients with premature ovarian insufficiency—our current state of knowledge. Int J Mol Sci. 2021;22(5):2594–2605. doi:10.3390/ijms22052594
[5] Lai D, Wang F, Chen Y, et al. Human amniotic fluid stem cells have a potential to recover ovarian function in mice with chemotherapy-induced sterility. BMC Dev Biol. 2013;13:34–47. doi:10.1186/1471-213X-13-34
[6] Kececi M, Akpolat M, Gulle K, et al. Evaluation of preventive effect of shilajit on radiation-induced apoptosis on ovaries. Arch Gynecol Obstet. 2016;293(6):1255–1262. doi:10.1007/s00404-015-3924-6
[7] Venkatesh S, Kumar M, Sharma A, et al. Oxidative stress and ATPase6 mutation is associated with primary ovarian insufficiency. Arch Gynecol Obstet. 2010;282(3):313–318. doi:10.1007/s00404-010-1444-y
[8] Agarwal A, Aponte-Mellado A, Premkumar BJ, et al. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol. 2012;10(10):49–117. doi:10.1186/1477-7827-10-49
[9] Kazemi A, Ramezanzadeh F, Nasr-Esfahani MH, et al. Does dietary fat intake influence oocyte competence and embryo quality by inducing oxidative stress in follicular fluid? Iran J Reprod Med. 2013;11(12):1005-1012.
[10] Zhao W-p, Wang H-w, Liu J, et al. Mitochondrial respiratory chain complex abnormal expressions and fusion disorder are involved in fluoride-induced mitochondrial dysfunction in ovarian granulosa cells. Chemosphere. 2019;215:619–625. doi:10.1016/j.chemosphere.2018.10.043
[11] Larsson N-G. Somatic mitochondrial DNA mutations in mammalian aging. Annu Rev Biochem. 2010;79(1):683–706. doi:10.1146/annurev-biochem-060408-093701
[12] Kumar M, Pathak D, Venkatesh S, et al. Chromosomal abnormalities & oxidative stress in women with premature ovarian failure (POF). Indian J Med Res. 2012;135:92–97. doi:10.4103/0971-5916.93430

[13] Hu W, Zhang Y, Wang D, et al. Iron overload-induced ferroptosis impairs porcine oocyte maturation and subsequent embryonic developmental competence in vitro. Front Cell Dev Biol. 2021;9:673291. doi:10.3389/fcell.2021.673291
[14] Qin X, Liang D, Hu M, et al. Chronic overload of concentration-dependent iron exerts different effects on ovarian function in C57BL/6J mice. Biol Reprod. 2021;104(6):1347–1359. doi:10.1093/biolre/ioab040
[15] Wang F, Liu Y, Ni F, et al. BNC1 deficiency-triggered ferroptosis through the NF2-YAP pathway induces primary ovarian insufficiency. Nat Commun. 2022;13(1):5871. doi:10.1038/s41467-022-33323-8
[16] Herraiz S, Buigues A, Díaz-García C, et al. Fertility rescue and ovarian follicle growth promotion by bone marrow stem cell infusion. Fertil Steril. 2018;109(5):908-918.e2. doi:10.1016/j.fertnstert.2018.01.004
[17] Zhang M, Xie T, Dai W, et al. Umbilical cord mesenchymal stem cells ameliorate premature ovarian insufficiency in rats. Evid Based Complement Altern Med. 2022;2022:9228456.
[18] Yan L, Wu Y, Li L, et al. Clinical analysis of human umbilical cord mesenchymal stem cell allotransplantation in patients with premature ovarian insufficiency. Cell Prolif. 2020;53(12):e12938. doi:10.1111/cpr.12938
[19] Song Y, Wang B, Zhu X, et al. Human umbilical cord blood–derived MSCs exosome attenuate myocardial injury by inhibiting ferroptosis in acute myocardial infarction mice. Cell Biol Toxicol. 2021;37(1):51–64. doi:10.1007/s10565-020-09530-8
[20] Yan X, Fu X, Jia Y, et al. Nrf2/Keap1/ARE signaling mediated an anti-oxidative protection of human placental mesenchymal stem cells of fetal origin in alveolar epithelial cells. Oxid Med Cell Longevity. 2019;2019:2654910.
[21] Chen X, Liang H, Xi Z, et al. BM-MSC transplantation alleviates intracerebral hemorrhage -induced brain injury, promotes astrocytes vimentin expression, and enhances astrocytes antioxidation via the Cx43/Nrf2/HO-1 Axis. Front Cell Dev Biol. 2020;8:302. doi:10.3389/fcell.2020.00302
[22] Luo Y, Lin X, Gong Y, et al. Rosenroot (Rhodiola): potential applications in aging-related diseases. Aging Dis. 2019;10(1):134–146. doi:10.14336/AD.2018.0511
[23] Kong Y-H, Xu S-P. Salidroside prevents skin carcinogenesis induced by DMBA/TPA in a mouse model through suppression of inflammation and promotion of apoptosis. Oncol Rep. 2018;39(6):2513–2526.
[24] Zhou L, Yao P, Jiang L, et al. Salidroside-pretreated mesenchymal stem cells contribute to neuroprotection in cerebral ischemic injury in vitro and in vivo. J Mol Histol. 2021;52(6):1145–1154. doi:10.1007/s10735-021-10022-0
[25] Ariyanti AD, Zhang J, Marcelina O, et al. Salidroside-pretreated mesenchymal stem cells enhance diabetic wound healing by promoting paracrine function and survival of mesenchymal stem cells under hyperglycemia. Stem Cells Transl Med. 2019;8(4):404–414. doi:10.1002/sctm.18-0143
[26] Yu P, Hu C, Meehan EJ, et al. X-Ray crystal structure and antioxidant activity of salidroside, a phenylethanoid glycoside. Chem Biodiversity. 2007;4(3):508-513. doi:10.1002/cbdv.200790043
[27] Hao X, Yuan J, Dong H. Salidroside prevents diabetes-induced cognitive impairment via regulating the Rho pathway. Mol Med Rep. 2019;19(1):678–684.
[28] Lu X, Bao H, Cui L, et al. hUMSC transplantation restores ovarian function in POI rats by inhibiting autophagy of theca-interstitial cells via the AMPK/mTOR signaling pathway. Stem Cell Res Ther. 2020;11(1):268. doi:10.1186/s13287-020-01784-7
[29] Doll S, Proneth B, Tyurina YY, et al. ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. Nat Chem Biol. 2017;13(1):91–98. doi:10.1038/nchembio.2239
[30] Barr RD. Adolescents, young adults, and cancer—the international challenge. Cancer. 2011;117(S10):2245–2249. doi:10.1002/cncr.26052
[31] Jeelani R, Khan SN, Shaeib F, et al. Cyclophosphamide and acrolein induced oxidative stress leading to deterioration of metaphase II mouse oocyte quality. Free Radical Biol Med. 2017;110:11–18. doi:10.1016/j.freeradbiomed.2017.05.006
[32] Cosgrove CM, Salani R. Ovarian effects of radiation and cytotoxic chemotherapy damage. Best Pract Res Clin Obstet Gynaecol. 2019;55:37–48. doi:10.1016/j.bpobgyn.2018.07.008
[33] Karska P, Matonog A, Sieradzka P, et al. Fresh insight into premature ovarian insufficiency. Ginekol Pol. 2021;92(7):518–524. doi:10.5603/GP.a2021.0111
[34] Lv X, Guan C, Li Y, et al. Effects of single and multiple transplantations of human umbilical cord mesenchymal stem cells on the recovery of ovarian function in the treatment of premature ovarian failure in mice. J Ovarian Res. 2021;14(1):119. doi:10.1186/s13048-021-00871-4
[35] Igboeli P, El Andaloussi A, Sheikh U, et al. Intraovarian injection of autologous human mesenchymal stem cells increases estrogen production and reduces menopausal symptoms in women with premature ovarian failure: two case reports and a review of the literature. J Med Case Rep. 2020;14(1):108–119. doi:10.1186/s13256-020-02426-5
[36] Bahrehbar K, Valojerdi MR, Esfandiari F, et al. Human embryonic stem cell-derived mesenchymal stem cells improved premature ovarian failure. World J Stem Cells. 2020;12(8):857–878. doi:10.4252/wjsc.v12.i8.857
[37] Mashayekhi M, Mirzadeh E, Chekini Z, et al. Evaluation of safety, feasibility and efficacy of intra-ovarian transplantation of autologous adipose derived mesenchymal stromal cells in idiopathic premature ovarian failure patients: non-randomized clinical trial, phase I, first in human. J Ovarian Res. 2021;14(1):5. doi:10.1186/s13048-020-00743-3
[38] Liu M-n, Zhang K, Xu T-m. The role of BMP15 and GDF9 in the pathogenesis of primary ovarian insufficiency. Hum Fertil. 2021;24(5):325-332. doi:10.1080/14647273.2019.1672107
[39] Li Q, Guo Y, Chen F, et al. Stromal cell-derived factor-1 promotes human adipose tissue-derived stem cell survival and chronic wound healing. Exp Ther Med. 2016;12(1):45–50. doi:10.3892/etm.2016.3309
[40] Tsubokawa T, Yagi K, Nakanishi C, et al. Impact of anti-apoptotic and anti-oxidative effects of bone marrow mesenchymal stem cells with transient overexpression of heme oxygenase-1 on myocardial ischemia. Am J Physiol Heart Circ Physiol. 2010;298(5):H1320–H1329. doi:10.1152/ajpheart.01330.2008
[41] Zhang M, Methot D, Poppa V, et al. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol. 2001;33(5):907–921. doi:10.1006/jmcc.2001.1367
[42] Haider HK, Ashraf M. Strategies to promote donor cell survival: Combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol. 2008;45(4):554–566. doi:10.1016/j.yjmcc.2008.05.004
[43] Das R, Jahr H, van Osch GJVM, et al. The role of hypoxia in bone marrow–derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng Part B Rev. 2010;16(2):159–168. doi:10.1089/ten.teb.2009.0296
[44] Ling L, Feng X, Wei T, et al. Effects of low-intensity pulsed ultrasound (LIPUS)-pretreated human amnion-derived mesenchymal stem cell (hAD-MSC) transplantation on primary ovarian insufficiency in rats. Stem Cell Res Ther. 2017;8(1):283. doi:10.1186/s13287-017-0739-3
[45] Chen X, Wang Q, Li X, et al. Heat shock pretreatment of mesenchymal stem cells for inhibiting the apoptosis of ovarian granulosa cells enhanced the repair effect on chemotherapy-induced premature ovarian failure. Stem Cell Res Ther. 2018;9(1):240. doi:10.1186/s13287-018-0964-4
[46] Yin Y, Liu D, Tian D. Salidroside prevents hydroperoxide-induced oxidative stress and apoptosis in retinal pigment epithelium cells. Exp Ther Med. 2018;16(3):2363–2368.
[47] Zhu Z, Li J, Zhang X. Salidroside protects against ox-LDL-induced endothelial injury by enhancing autophagy mediated by SIRT1-FoxO1 pathway. BMC Complement Altern Med. 2019;19(1):111. doi:10.1186/s12906-019-2526-4
[48] Dixon SJ Lemberg K, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012;149(5):1060-1072. doi:10.1016/j.cell.2012.03.042
[49] Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. Cell. 2017;171(2):273-285. doi:10.1016/j.cell.2017.09.021
[50] Zhang J, Liu Y, Yao W, et al. Initiation of follicular atresia: gene networks during early atresia in pig ovaries. Reproduction. 2018;156:23-33. doi:10.1530/REP-18-0058
[51] Du R, Cheng X, Ji J, et al. Mechanism of ferroptosis in a rat model of premature ovarian insufficiency induced by cisplatin. Sci Rep. 2023;13(1):4463. doi:10.1038/s41598-023-31712-7
[52] Chen X, Yu C, Kang R, et al. Iron metabolism in ferroptosis. Front Cell Dev Biol. 2020;8:590226. doi:10.3389/fcell.2020.590226

[53] Jiang X, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology and role in disease. Nat Rev Mol Cell Biol. 2021;22(4):266–282. doi:10.1038/s41580-020-00324-8
[54] Zeng B, Zhang P, Zheng M, et al. Detection and identification of the oxidizing species generated from the physiologically important Fenton-like reaction of iron(II)-citrate with hydrogen peroxide. Arch Biochem Biophys. 2019;668:39–45. doi:10.1016/j.abb.2019.05.006
[55] Geng Z, Nie X, Ling L, et al. Electroacupuncture may inhibit oxidative stress of premature ovarian failure mice by regulating intestinal microbiota. Oxid Med Cell Longevity. 2022;2022:4362317.
[56] Chen Y, Zhao Y, Miao C, et al. Quercetin alleviates cyclophosphamide-induced premature ovarian insufficiency in mice by reducing mitochondrial oxidative stress and pyroptosis in granulosa cells. J Ovarian Res. 2022;15(1):138. doi:10.1186/s13048-022-01080-3
[57] International BR. Retracted: preventive electroacupuncture alleviates oxidative stress and inflammation via Keap1/Nrf2/HO-1 pathway in rats with cyclophosphamide-induced premature ovarian insufficiency. BioMed Res Int. 2023;2023:9816950. doi:10.1155/2023/9816950
[58] Wang H, He Y, Cheng D, et al. Cypermethrin exposure reduces the ovarian reserve by causing mitochondrial dysfunction in granulosa cells. Toxicol Appl Pharmacol. 2019;379:114693. doi:10.1016/j.taap.2019.114693
[59] Wang D, Zhang S, Ge X, et al. Mesenchymal stromal cell treatment attenuates repetitive mild traumatic brain injury-induced persistent cognitive deficits via suppressing ferroptosis. J Neuroinflammation. 2022;19(1):185. doi:10.1186/s12974-022-02550-7
[60] Lin F, Chen W, Zhou J, et al. Mesenchymal stem cells protect against ferroptosis via exosome-mediated stabilization of SLC7A11 in acute liver injury. Cell Death Dis. 2022;13(3):271. doi:10.1038/s41419-022-04708-w
[61] Xu J, Zhang M, Liu F, et al. Mesenchymal stem cells alleviate post-resuscitation cardiac and cerebral injuries by inhibiting cell pyroptosis and ferroptosis in a Swine Model of cardiac arrest. Front Pharmacol. 2021;12:793829. doi:10.3389/fphar.2021.793829
[62] Chen X, Kang R, Kroemer G, et al. Broadening horizons: the role of ferroptosis in cancer. Nat Rev Clin Oncol. 2021;18(5):280–296. doi:10.1038/s41571-020-00462-0
[63] Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev. 2006;38(4):769–789. doi:10.1080/03602530600971974
[64] Maiorino M, Conrad M, Ursini F. GPx4, lipid peroxidation, and cell death: discoveries, rediscoveries, and open issues. Antioxid Redox Signal. 2018;29(1):61–74. doi:10.1089/ars.2017.7115
[65] Ingold I, Berndt C, Schmitt S, et al. Selenium utilization by GPX4 is required to prevent hydroperoxide-induced ferroptosis. Cell. 2018;172(3):409-422.e21. doi:10.1016/j.cell.2017.11.048
[66] Zhang H-L, Hu B-X, Li Z-L, et al. PKCβII phosphorylates ACSL4 to amplify lipid peroxidation to induce ferroptosis. Nat Cell Biol. 2022;24(1):88–98. doi:10.1038/s41556-021-00818-3