Dexmedetomidine is Resistant to Ferroptosis by Activating mTOR-TFR1 Signaling

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Research Article

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Abstract

Background: With the development of society, Neurodegenerative disease (ND), such as alzheimer's disease, is more and more important to the researchers. Metal iron may play a crucial role in this disease, so our research constructed the iron overloading model in nerve cells, induce the ferroptosis, simulate the state of the nerve in the body, and used the anesthesia Dexmedetomidine (Dex), and study whether the Dex can inhibit the ferroptosis and reduce the ND.

Methods: Cell proliferation kit CCK8 and PI/Hoechst fluorescence double staining were used to detect the proliferation and apoptosis of HT22 cells. Western blot (WB) was used to detect the expression of PTGS2 and ACSL4, pathway proteins mTOR, TFR1. ROS content in HT22 cells was determined by DHE fluorescence probe. Lipid Peroxidation in nerve cells was detected by MDA Assay. Mito-ferrorange fluorescent probe was used to detect the level of ferrous ions in cells to demonstrate that ferroptosis occurred in nerve cells and Dex could protect nerve cells from ferroptosis.

Results: Dex inhibits ferroptosis by regulating the mTOR-TFR1 pathway, reducing lipid peroxidation, intracellular reactive oxygen accumulation (ROS), reducing iron ions, and alleviating mitochondrial damage. mTOR is a well-known autophagy target and has been found to be closely related to ferroptosis. Dex activates the mTOR pathway, inhibits iron entry into the cell, reduces iron influx, and prevents ferroptosis by fenton reaction between excessive iron and lipids in the cell.

Conclusion: Dex protects nerve cells from ferroptosis by regulating the mTOR-TFR1 pathway.

Introduction

At this stage, the global aging trend is increasing. Neurodegenerative disorders are a type of age-dependent diseases that often occur in the elderly, but they are beginning to be younger. For instance, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, lateral sclerosis of the spinal cord. With the further development of society, patients with neurodegenerative diseases (such as Alzheimer's disease, Parkinson's disease, etc.) have received more and more attention, and the further mechanisms and treatment and prevention measures of these diseases have gradually attracted the attention of researchers. Some studies believe that the increase of iron content in the brain is an important factor in causing neurodegenerative diseases[1, 2]. In the microglia and astrocytes of the cortex, cerebellum, hippocampus, basal ganglia, and amygdala, iron deposits histochemically detected generally increase with age[3]. These studies suggested that increased iron content in the brain may affect nerve cell function, and excessive iron may cause neurodegenerative diseases. Therefore, in our experiment, we use ferrous ammonium citrate (FAC) to induce HT22 cells to simulate a neurodegenerative disease model to explore the therapeutic effect of dexmedetomidine on iron-induced neurodegenerative disorders.

Ferroptosis, an iron-dependent, lipid peroxide accumulates to toxic level result in cell death which is different from apoptosis, closely associated with neuronal aging. As a new style of programmed cell

death, ferroptosis has many characters which are different from general cell death. For example, a concentration of mitochondrial membrane in cell which has occurred ferroptosis, mitochondrial morphological change is the decreased or disappeared mitochondria cristae and mitochondrial membrane ruptured. A role for mitochondria in regulating ferroptosis is contentious, its regulatory effect may be related to the production of ROS[4]. Mechanically, we can lead ferroptosis from three main molecules aspects[5]. The first aspect, system xCT^- regulates glutathione peroxidase 4 (GPX4) and glutathione to impact ferroptosis, therefore through reducing the levels of GPX4 and glutathione (e.g., erastin, RSL3) to induce ferroptosis. The second aspect, by inducing iron overload to produce excessive Fe^{2+} and consequently stimulate the Fenton reaction which could produces an abundance of reactive oxygen species (ROS) and lipid peroxidation, and molecules of the third aspect work through the directly generation of ROS and lipid peroxide. In our experiment, we used FAC to induce iron overload and then trigger ferroptosis, discussed the relative mechanisms. iron as an important mental substance in the growth and development of the body, and it has a crucial participant in ferroptotic cells. Whether excessive iron is the key to ferroptosis. A large amount of iron accumulates outside the cells to induce iron overload, the extracellular iron was combined with transferrin (TF) which is on the membrane, to form a complex and subsequently the complex was transported into the cells through the transferrin receptor 1 (Transferrin receptor1, TFR1) in endocytosis. Fe^{3+} is reduced to toxic Fe^{2+} by intracellular iron reductase, moreover, intracellular unstable iron pool accumulated excessive Fe^{2+} as a raw material to trigger ferroptosis as well as induce ferroptosis in due time[6, 7].

The mechanistic target of rapamycin kinase (mTOR) is a classical autophagy-reliant regulatory protein, it can affect the pathway of autophagy, inflammation and apoptosis. It has been affirmed that the classical autophagy inducer rapamycin can inhibit the activation of mTOR and then promote autophagy and ferroptosis. Studies [8, 9] have confirmed that mTOR can regulate iron and affect the metabolism of iron ions. The reports [10] that mTOR can regulate ferroptosis to remit cell death, the specific mechanism that mTOR regulates ferroptosis is not clearly. It has been shown that mTOR can modulate the stability of Transferrin receptor 1(TFR1) and subsequently play a part for a balanced iron homeostasis [11]. Hence, we assumed that activating mTOR can influence the production of ferroptosis and subsequently relieve neuronal cells death. Dexmedetomidine is a high selective α2-adrenergic receptor (α2-AR), it not only restrains the activity of sympathetic nerves via analgesic, reduce inflammation[12, 13], but also exerts the obvious protective effects on many significant organs, for instance, the heart, brain and kidney. There is experimental proof that Dexmedetomidine possessed neuroprotective effects against diversified brain injury but underlying mechanisms still remain elusive[14, 15]. In vivo experiments[16] have proved that Dex has a protective effect within a certain concentration range, and the excess will increase the toxicity of nerve cells. Therefore, we conducted a concentration test of Dex to ensure its protective effect. In vitro studies have demonstrated that dexmedetomidine affects the enginery of some neuronal cells such as microglia and astrocytes. Though Dexmedetomidine has apparent protective role in brain protection, it is not yet clarified whether Dexmedetomidine can protect neuronal cells from ferroptosis upon iron overload. Even though the detailed mechanism of these phenomena is unclear, according to clinical reports, Systemic administration of Dexmedetomidine can ameliorate neurocognitive functions as well as

alleviate neuroinflammation after other illnesses including surgery. However, whether Dex has an interventional effect on FAC-induced neurotoxicity has yet to be proven. In this study, we sought to test for the protective effect of Dex on the FAC-triggered dysfunction of ferroptosis, a key step associated with neurotoxicity. We also assessed the impact of dexmedetomidine on iron accumulation and mobilization. In general, Dexmedetomidine has prominent superiority in neuroprotection, however, whether Dexmedetomidine can play a neuroprotective effect by regulating ferroptosis and how to modulate ferroptosis is ambiguous. At present, experiments have concluded that Dex can affect cognition by regulating iron metabolism[17]. In our studies, our data confirmed that Dexmedetomidine which mainly activates mTOR-TFR1 pathway, and ultimately play a neuroprotective effect. These findings may offer new insight into cell death which was induced by ferroptosis. Dexmedetomidine may safeguard neuronal cells from ferroptosis via regulating iron overload maintain iron homeostasis.

Materials And Methods

Reagents and antibodies

Dexmedetomidine was purchased from Yangtze River Pharmaceutical (Group) Co., Ltd., rabbit anti-ACSL4, rabbit anti-TFR1, and rabbit anti-mTOR were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-phospho-p-mTOR, anti-β-actin, and anti-PTGS2 antibodies were purchased from Abclonal, WuHan. Goat anti-rabbit immunoglobulin (IgG) horseradish peroxidase (HRP) secondary antibodies were purchased from Abclonal, (Wuhan, China). mTOR, TFR1, ACSL4, PTGS2, and GRAPDH primers were synthesized by Anhui tongyong Biological and Technological Company (Anhui, China).HT22 dedicated complete media was obtained from Procell Life Science&Technology Co., Ltd. (Wuhan, China).

Cell culture and treatment

The mouse hippocampal neuron cell line was purchased from Procell Life Science&Technology Co., Ltd. (Wuhan, China). The cell line was cultured in HT22 dedicated complete media in a cell incubator with 37°C□5%CO2 and 95% humidity. All neuronal cells were used within 20 generations. Selecting well-growing cells are used in experiments. Inoculate the cells in culture plate at appropriate cell density (in general converge to 80%-90%) and incubated a period of time in a cell incubator, and then subjected to treatments as described in each experiment.

Cell Viability Assay

Accordance with the reagent manufacturer’s protocol, we used CCK-8 Kit to detect the level of cell viability in different concentration of Dexmedetomidine at the same time, the absorbance values were measured at 450 nm using a microplate reader.

PI/Hoechst Fluorescence double-labeling method to detect cell apoptosis

Divided the HT22 cells into several groups as required and seeded cells on a six-well plate at a density of 1×10^5 cells/well. After culturing for 12 hours, added Hoechst 33342 and PI solution at a final concentration of 5mg/L to the medium. incubated for 10 minutes in the 37°C, 5%CO_2 cell incubator. Used fluorescent microscope to observe and calculate the relative cell survival rate.

Malondialdehyde (MDA) Assay

Malondialdehyde (MDA), an end product of lipid peroxidation. Analysis of lipid peroxidation was executed by quantification of Lipid Peroxidation MDA Assay Kit with a specific colorimatria kit (cat #ab118970; Abcam) following reagent manufacturer's illustrations. Treated the cells according to the instructions, then the absorbance of cells was detected with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 532 nm, with MDA concentration calculated based on a standard curve.

Lipid ROS quantification

Configure PBS containing DHE fluorescent dye: add DHE fluorescent dye with a final concentration of 10μmol/L to PBS, inoculated HT22 cells in a six-well plate at an appropriate density as required, cultured for 12 hours, then added 200μM FAC to medium and subsequently incubated 24 hours, cells were stained with DHE for 30 min after washed twice with PBS. Used fluorescent microscope to observe and calculate the relative percentage of reactive oxygen cells.

Ferrous ion Assay

The level of Fe^{2+} was measured using Mito-FerroOrange (Dojindo Molecular Technologies, Tokyo, Japan). In brief, cells were seeded on Confocal small dishes and subjected to different treatments. The supernatant was discarded, and the cells were washed three times with HBSS. Then, the Mito-FerroOrange working solution (2 μL, 2mL) was added to the cells and incubated for 30 min. The cells were observed by confocal fluorescence microscopy.

Mitochondrial morphology and structure detection

Inoculate HT22 cells in a culture flask at an appropriate density, culture for 12h until the cells converge to 80%-90%, cells were incubated for 12 hours after indicated treatment and then extracted the electron microscope samples. Place samples under a microscope to observe the change of mitochondrial cristae, mitochondrial membrane density and cell nucleus of different groups.

The western blot Analyses

Protein expression was measured by Western blot in accordance with standard agreements. After indicated treatment and incubated, cells were acquired and washed twice with PBS and lysed in RIPA buffer including complete protease and phosphatase inhibitor. Following the instructions, the concentration of protein was quantified by the bicinchoninic acid protein assay kit. Boiled proteins were separated with 6%-10 % polyacrylamide gel electrophoresis (SDS-PAGE) and afterwards transferred onto

polyvinylidene fluorides (PVDF) membrane through Semi-dry electroblotting. After blocking with 5 % skim milk, membranes were probed overnight at 4°C with specific primary antibodies. Subsequently, washing with TBST, membranes were incubated with an HRP-conjugated secondary antibody for 1h at room temperature. Enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA) was used to measure the expression of target bands. The primary antibodies used were as follows: anti-PTGS2, anti-p-mTOR, anti-mTOR, anti-TFR1.

Statistical analyses

GraphPad Prism software 8.0 were employed to statistical in this experiment. Data from three independent experiments and presented as mean ± SD. All data are depicted as the mean standard error of the mean (SEM). Differences between two groups and among groups were analyzed using Student's t-test and one-way ANOVA. $p<0.05$ was regarded as statistically significant.

Results

FAC significantly supressed the proliferation of HT22 cell lines in vitro.

According to the literature [2, 17, 18, 19], we found that the most appropriate FAC concentration for HT22 cells was 150 μM and incubated for 24h. This concentration was used as the stimulus concentration for subsequent experiments. To further verify whether the inhibition of cell proliferation induced by FAC was caused by ferroptosis, we used ferroptosis inhibitor ferrostain-1, and our experiment verified that FAC could indeed induce ferroptosis. According to our experimental results, the levels of ferroptosis marker proteins ACSL4 and PTGS2 in the FAC treatment group were significantly higher than those in the control group (Fig. 1A), and the Fe^{2+} probe, lipid peroxidation and ROS were generated as expectations (Fig. 1B-D), suggesting that FAC did inhibit the proliferation of HT22 cell lines and induce ferroptosis.

Dex has no cytotoxicity within a certain range.

To confirm whether the antioxidative protection due to Dex is connected to the regulation of iron metabolism, the effect of Dex treatment on FAC-induced oxidative stress was evaluated. As CCK-8 test described, 1 μM and 5 μM Dex promoted the viability of HT22 cells (Fig. 2A), whereas the effect of 25 μM Dex was not significant. We chose a dose of 5 μM Dex. The selected dose of Dex was referred to the literature[20, 21]. This is consistent with the conclusions of the previous references. FAC decreased the viability of HT22 cells (Fig. 2B), but pretreatment with 1 μM and 5 μM Dex prevented the reduction in HT22 cell viability ($P<0.05$ vs. HT22 group), in a dose-dependent manner. Then we performed PI/Hochest for cell apoptosis and found that the cell survival rate of the 5μM Dex group was significantly higher than that of the FAC group. All the above conclusions suggest that Dex has protective effect on cells in appropriate dose range.

Dex alleviates iron overload and the accumulation of ROS which induced by ferroptosis

To confirm whether Dex arrested ferroptosis in HT22 cells, we firstly assayed FAC-induced changes in cellular ferrous irons. After processing with Fe^{2+} probe, we found that the levels of iron were decreased following Dex treatment (Fig. 3A). In addition, Dex treatment reduced the level of MDA (Fig. 3B) in HT22 cells. The characteristic of mitochondria in Ferroptosis is that the mitochondrial

membrane thickens and the membrane density increases, which causes the mitochondria to depolarize [18], ferroptosis is also accompanied by increased autophagosomes [19]. We carried out the detection of intracellular ultrastructure (mitochondria and nucleus). The results are shown in the figure (Fig. 3C). The morphology of mitochondria has changed, but the nucleus has not changed. As shown in the western blot picture (Fig. 3D), the levels of the Ferroptosis marker proteins ASCL4 and PTGS2 in the FAC treatment group compared with Dex group were significantly increased. The results of DHE probe detection of ROS also indicate that Dex has an inhibitory effect on FAC-induced ferroptosis (Fig. 3E). These consequences indicated that Dex was able to restrain ferroptosis in HT22 cells. These

data indicate that Dex contributed to rescue FAC-induced Ferroptosis in HT22 cells.

Dex regulates Ferroptosis through activating mTOR/TFR1 pathway

Subsequently, we examined where Dex could regulate Ferroptosis in HT22 cells., explore the way that Dex regulates Ferroptosis. In present study, we

found that Dex mainly modulated the iron homeostasis. Our experimental results show that

after treatment with Dex, the protein expression and mRNA levels of TFR1 are significantly reduced, and TFR1 is at the entrance of iron metabolism, so you can grasp the amount of iron ions entering the cell. Probe Fe^{2+} result showed that the Dex treatment group is the same as the Fer-1 group, and the Fe^{2+} is significantly reduced. It has clarified that Dex can regulate the metabolism of iron. mTOR signaling is tightly associated with regulation of autophagy and our consequence implied that Dex was able to modulate mTOR signaling to regulate Ferroptosis through maintain iron homeostasis. In our study, Dex was found to promote the phosphorylation of mTOR while suppress the express of TFR1 (Fig. 3F). It suggests that Dex may regulate iron homeostasis by regulating mTOR to inhibit TFR1.

Discussion

We have previously testified that Dex pretreatment can provide neuroprotection against glutamate

damage in HT22 cells. On the basis of these findings,

the present study further examined whether Dex could ameliorate nerve damage and cognitive impairment and explored the potential mechanisms in Dex alleviate iron overload-induce ferroptosis. Our main findings include the following: (1) both pre- and posttreatment with Dex alleviated iron overload-induced cognitive impairment and nerve damage ;(2) the neuroprotective benefits of Dex

cultured hippocampal neurons were linked to suppression of the ferroptosis and then attenuation of oxidative stress; (3) Dex treatment prevented FAC-mediated iron overload and ferroptosis-

induced neuronal damage; (4) promoting mTOR activation might contribute to the antioxidant effects of dexmedetomidine in the developing hippocampus following iron overload.

There are many ways of death in the growth and development of the body, such as apoptosis, necrosis, autophagy, and ferroptosis. In our experiment, we explored a series of problems caused

by ferroptosis. This is a relatively new way of death found so far, and it is closely related to mitochondria. The latest evidence shows that ferroptosis is involved in mitochondria, and the three main metabolic pathways of ferroptosis in cells also occur in mitochondria. With the number of Neurodegenerative diseases patients increasing, Dex could be a new alternative in improving symptoms for advanced Neurodegenerative diseases patients. Like many people, we believe that Ferroptosis is an important factor in neurodegenerative diseases. Iron content in neurodegenerative diseases itself will increase, and oxidative stress will also occur, which promotes Ferroptosis. In our study, iron agents were used to directly induce the overload of the stickers to construct an iron death model, we show that ferroptosis can also be induced by FAC. Ferroptosis which induced by FAC mainly through regulating iron homeostasis, increase the amount of iron accumulation at the entrance of TFR1, to make TFR1 open, a large amount of iron flows into the cell, promotes the occurrence of a Fenton reaction to generate lipid peroxyl radicals (PUFA-OO•), and ultimately form lipid hydroperoxides (PUFA-OOH), accelerates lipid accumulation. Eventually causes ferroptosis. Iron is an important participant in the

Fenton reaction. Our experiments manifest that FAC induces ACSL4 expression, which is vital for regulating the biosynthesis of PUFA-PLs (the type

of lipids that are susceptible to peroxidation). Indexes related to ferroptosis (TFR1, Fe^{2+}) increased significantly. The results indicate that the iron homeostasis has been broken by FAC. It can be seen that iron overload is associated with lipid metabolism and jointly regulates ferroptosis.

Our findings here revealed that Dex suppressed ferroptosis and promoted cell proliferation through activating mTOR-TFR1 pathway. In the presupposition research, the levels of TFR1, Fe^{2+}, MDA, and lipid ROS were upregulated, but the mTOR levels were downregulated by FAC. These results confirmed that FAC-induced ferroptosis can be suppressed by Dex. As a commonly used clinical anesthetic, Dex has good use value in the prevention and treatment of neurodegenerative diseases. In our experiment, after treatment with Dex, the ferroptosis index induced by FAC has decreased significantly, and the morphology of cells under the microscope, mitochondrial cristae and mitochondrial membrane density all recovered to a certain extent compared with the model group. This shows that Dex can regulate neurodegenerative diseases. Our next experiment proved that Dex can improve nerve damage by regulating mTOR, and further confirmed that Dex can regulate iron metabolism and improve neurodegenerative diseases. Dex can affect iron homeostasis by regulating mTOR-TFR1 pathway, inhibit ferroptosis, and improve Degenerative diseases. Of note, our key finding showing that Dex modulates iron homeostasis through

mTOR-TFR1 signaling pathway is in line with recent evidence documenting. mTOR is often used to carry out related research autophagy, Dex inhibits ferroptosis has not yet been examined by activating mTOR-TFR1, so this finding needs further depth to demonstrate in vivo experiments. This paper prevention and treatment of neurodegenerative diseases provides new ideas, but also to provide new guidelines for the clinical use of narcotic drugs, especially the potential role of Dex repairs nerve damage, but also extends the range for the study of ferroptosis, it is not a single way to die, it is always complementary to other ways cell death, or primary or secondary auxiliary guide, ultimately leading to cell death.

Number of compounds have been found to trigger ferroptosis and apoptosis simultaneously. We verified that the FAC-induced cell death inhibited by Dex has feroptosis. At this stage, the way that Dex inhibits nerve damage by regulating ferroptosis has not been clearly confirmed. Most studies have been studying Dex to protect nerve cells from damage by regulating autophagy or apoptosis. Our research provides a new understanding and direction for narcotic sedatives to regulate ferroptosis and exert neuroprotective effects.

There were several limitations in our study. First, there is no in vivo experiments, but simply explained the problem from the perspective of the cell, a certain lack of reliability is not precise enough, the need for further in vivo experiments to further verification. Next, It simply proved Dex's action pathway from the aspect of protein gene, and did not carry out the next step of gene knockout to verify Dex's specific action target. this is a possible future work for our research.

Conclusions

In summary, this study revealed that the neuroprotection of Dex is partly related to the inhibition of intracellular iron accumulation induced by FAC. The regulatory effect of Dex on iron metabolism is linked to the regulation of iron importer and exporter through its effect on mTOR/TFR1 signaling.

The study was approved by Anhui Provincial laboratory of inflammatory and immunity disease, our all methods were performed in accordance with the relevant guidelines and regulations by Availability of data and materials. If you want to request the data, please contact me.

Abbreviations

ND:Neurodegenerative disease; Dex: Dexmedetomidine; ROS: reactive oxygen accumulation; GPX4: glutathione peroxidase 4; qPCR: Quantitative real-time polymerase chain reaction; mTOR: The mechanistic target of rapamycin kinase; TFR1: Transferrin receptor 1TFR1; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Declarations

Declaration of Competing Interest

I confirm that the methods used in this experiment are implemented with the approval of the Ethics Committee of Chaohu Hospital affiliated to Anhui Medical University. All authors have taken part in this study. They all declared that they have anything to disclose regarding funding from industries or conflict of interest as for the manuscript.

Acknowledgement

None

Authors' contributions

HD participated in the design of the experiment, methodology, data analysis, and drafted the manuscript. JYW participated in the collection of data and analysis for the work, carried out the statistical analysis. YH provided the experimental site and partial technical support, while YHL provided experimental guidance All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

The design of this protocol follows the tenets of the Declaration of Helsinki, approval to conduct the experiment was obtained from the Medical Ethics Committee of Chaohu Hospital affiliated to Anhui Medical University, Anhui (ethics number: KYXM-202111-009).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Figures

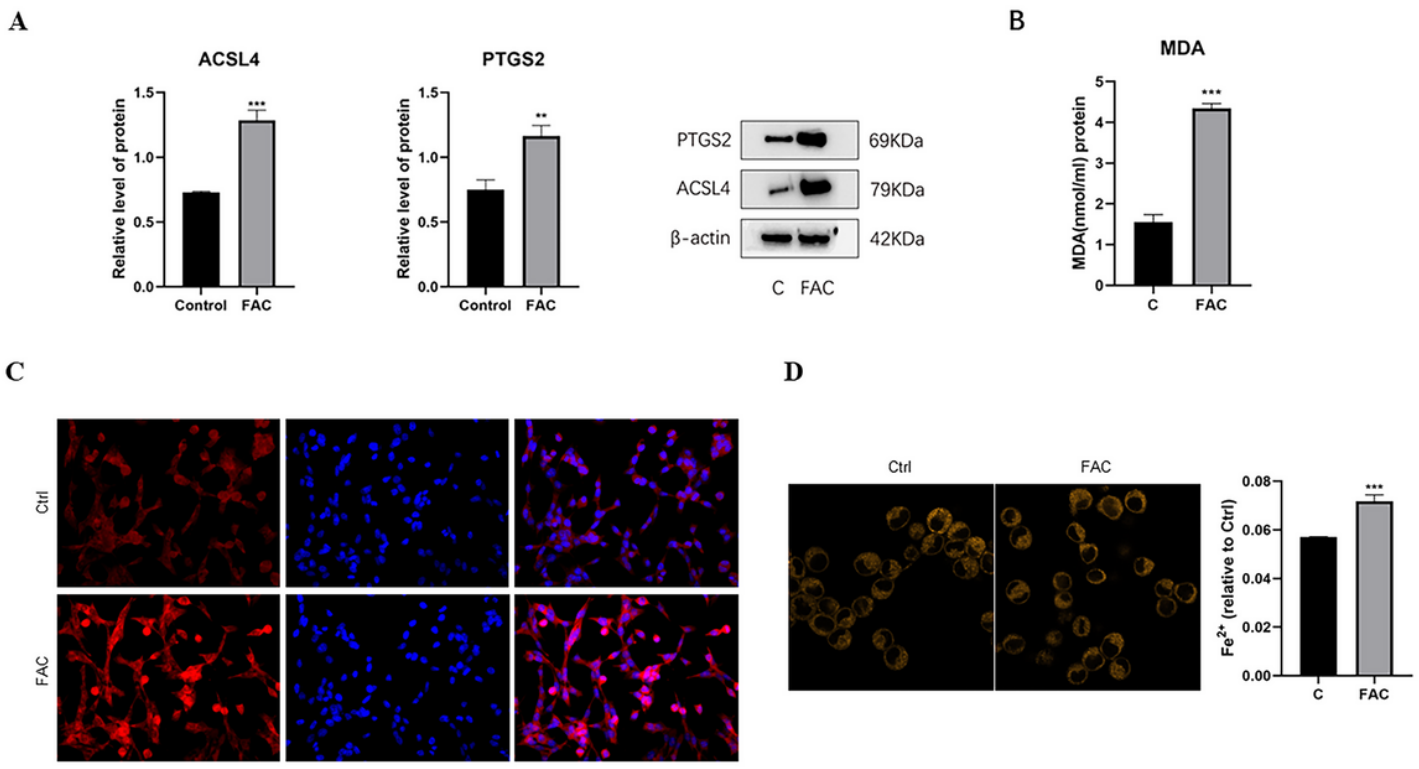

Figure 1

FAC significantly inhibited the proliferation of HT22 cell lines in vitro. A Western blot analysis of ACSL4 and PTGS2 protein levels in HT22 cells following treatment with FAC for 24 h. B testing Lipid peroxidation degree using MDA kit after FAC treatment. C using ROS probe detects ROS levels in cells treated by different methods. D Measuring cell fluorescence strength by Fe2+ staining in indicated HT22 cells

following treatment with FAC for 12h. The second panel showed a representative image of Fe2+ staining in HT22 cells after FAC treatment. *P<0.05, **P<0.01, and ***P<0.001

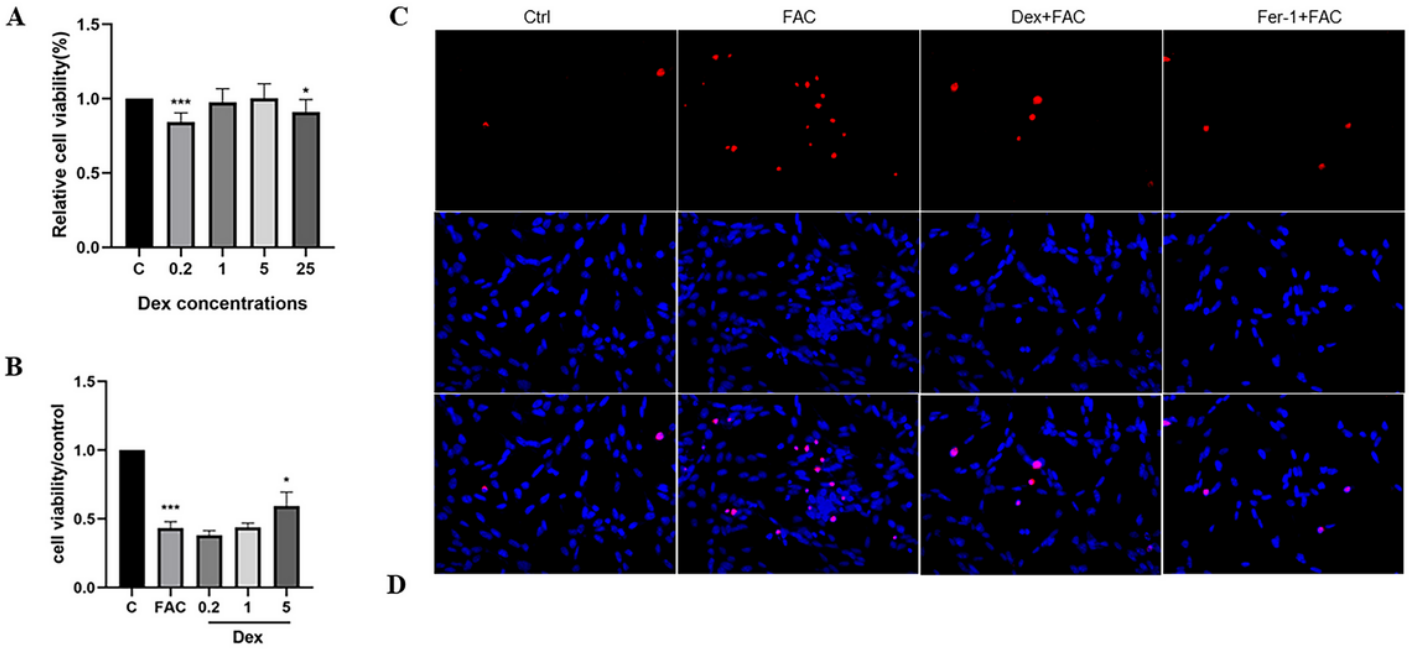

Figure 2

Dex arrests FAC-induced Ferroptosis and cytotoxicity. A Cell viability was tested 12h after the Dex（0.2μM, 1μM、5μM、25μM） treatments using CCK-8 kit. B The cells were subsequently treated with FAC. C PI/Hoechst Fluorescence double-labeling method to detect cell apoptosis. *P<0.05, **P<0.01, and ***P<0.001

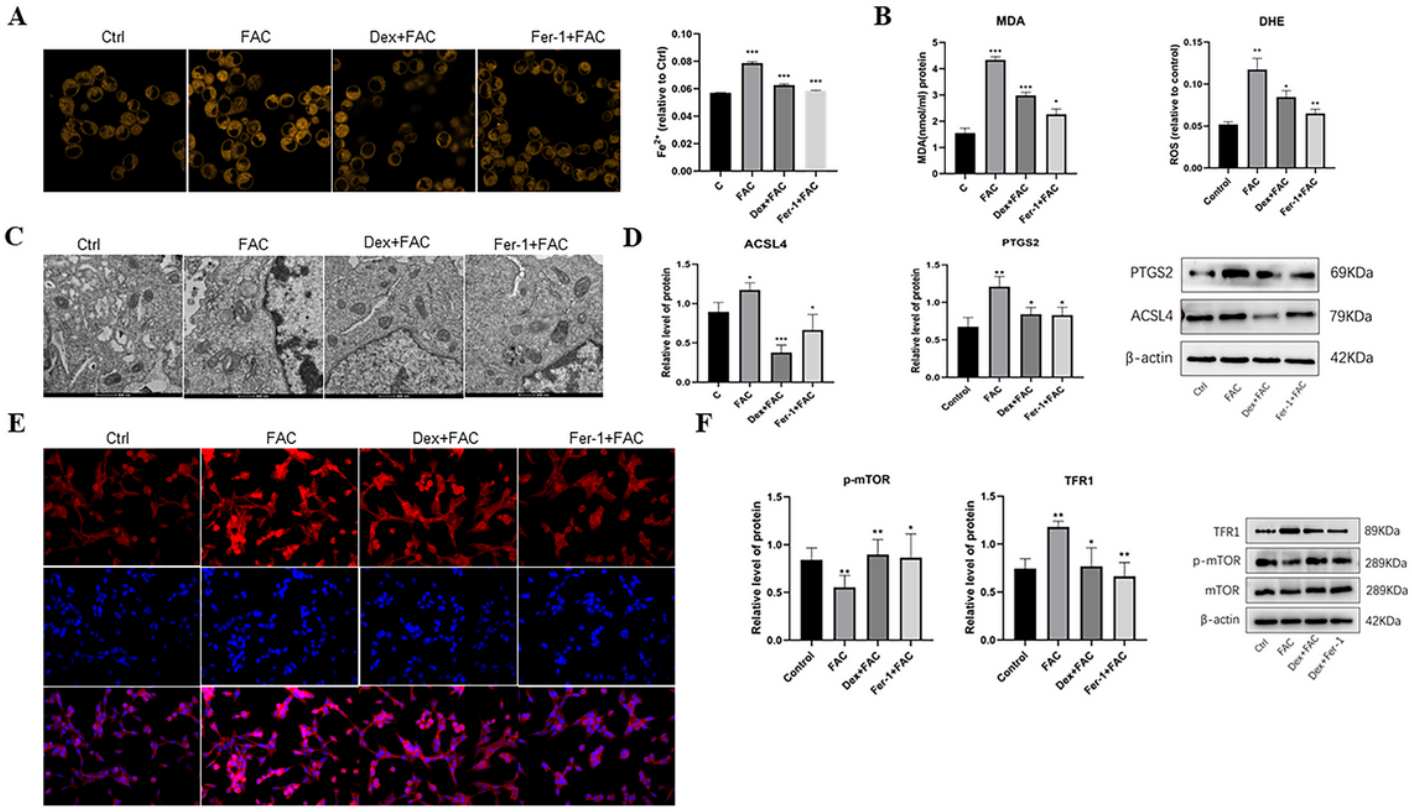

Figure 3

Dex alleviates iron overload and the accumulation of ROS which induced by ferroptosis. A Measuring cell fluorescence strength by Fe2+ staining in indicated HT22 cells following treatment with FAC for 12 h. B testing Lipid peroxidation degree using MDA kit after FAC treatment. C Electron microscopy shows the ultrastructure of mitochondria with or without Dex after FAC treatment. D Western blot analysis of ACSL4 and PTGS2 protein levels in iron-overloaded HT22 cells following treatment with Dex for 12h. E using ROS probe detects ROS levels in cells treated by different methods. F Dex regulates Ferroptosis through activating mTOR/TFR1 pathway. Changes in protein and gene levels of mTOR/TFR1 pathway after Dex treatment *P<0.05, **P<0.01, and ***P<0.001