Research Article

Ebselen, Iron Uptake Inhibitor, Alleviates Iron Overload-Induced Senescence-Like Neuronal Cells SH-SY5Y via Suppressing the mTORC1 Signaling Pathway

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Increasing evidence highlights that excessive iron accumulation in the brain plays a vital role in neuronal senescence and is implicated in the pathogenesis of age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). Therefore, the chemical compounds that eliminate an iron overload may provide better protection against oxidative stress conditions that cause the accumulation of senescent cells during brain aging. Ebselen has been identified as a strongly useful compound in the research on redox biology mechanisms. We hypothesized that ebselen could alleviate an iron overload-induced oxidative stress and consequently reverses the senescence-like phenotypes in the neuronal cells. In the present study, SH-SY5Y cells were treated with ferric ammonium citrate (FAC) before ebselen, and the evaluation of the cellular iron homeostasis, the indicators of oxidative stress, and the onset of senescence phenotypes and mechanisms were carried out accordingly. Our findings showed that ebselen ameliorated the FAC-mediated iron overload by decreasing the expression of divalent metal transporter 1 (DMT1) and ferritin light chain (FT-L) proteins. In contrast, it increased the expression of ferroportin 1 (FPN1) protein and its correlation led to a decrease in the expression of the cytosolic labile iron pool (LIP). Furthermore, ebselen significantly reduced reactive oxygen species (ROS) and rescued the mitochondrial membrane potential (ΔΨm). Notably, ebselen restored the biomarkers of cellular senescence by reducing the number of senescence-associated β-galactosidase (SA-β-gal) positive cells and senescence-associated secretory phenotypes (SASP). This also suppressed the expression of p53 protein targeting DNA damage response (DDR)/p21 cyclin-dependent kinase (CDK) inhibitor through a mTORC1 signaling pathway. Potentially, ebselen could be a therapeutic agent for treating brain aging and AD by mitigating iron accumulation and restoring senescence in SH-SY5Y cells.

1. Introduction

Iron is an essential trace element for normal brain physiological functions such as oxygen transportation, cellular metabolism, myelination, and neurotransmitter synthesis [1]. Iron is transported from the circulatory system into the brain parenchymal tissue through the blood-brain barrier (BBB), primarily mediated by either the transferrin/transferrin receptor (Tf/TfR) or the divalent metal transporter 1 (DMT1) pathways. These mechanisms predominantly take place within the brain microvascular epithelial cells (BMVECs) [2, 3]. Neurons acquire iron through DMT1, a key player in a nontransferrin-bound iron (NTBI), which facilitates ferrous iron (Fe^{2+}) uptake from the plasma membrane and its subsequent transportation from endosomes into the cytosolic labile iron pool (LIP). Notably, this process is altered within the context of the aged brain [4]. However, the accumulation of excessive iron in the brain can

initiate the Fenton reaction, resulting in the generation of hydroxyl free radicals. Given the relatively lower levels of antioxidant defenses, this process can trigger downstream events that eventually lead to cell death [5].

Brain iron overload is the major function of aging and plays an important role in the pathogenesis of age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), although the molecular mechanisms underlying iron-induced neurodegeneration remain an ongoing examination [6]. Hence, the maintenance of cellular iron homeostasis must be tightly regulated by a multitude of iron uptake (TfR and DMT1), iron storage (ferritin), iron export (ferroportin 1; FPN1), and its ligand, the master iron-regulatory hormone hepcidin. These mechanisms concomitantly occur to ensure the normal physiological levels of cytosolic LIP for the cellular requirements and permit the safe handling of iron in the brain [6–8]. Recent studies have demonstrated that the increase in the NTBI pool is coupled with the neuroinflammation response to proinflammatory cytokines release and is considered to increase brain aging and several neurodegenerative diseases [9]. The mammalian target of rapamycin (mTOR) is an evolutionarily conserved Ser/Thr protein kinase that belongs to the phosphatidylinositol-3 kinase- (PI3K-) related kinase family. The mTOR is present as a catalytic subunit in two protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [10, 11]. The mTOR pathway is a central regulator of cellular metabolism and physiology, with important roles in cell growth and proliferation [12, 13]. Although the involvement of the mTOR pathway in regulating cellular senescence and aging has been studied extensively, the mechanism through which this occurs is still unclear. Furthermore, many of the processes controlled by mTORC1 are dependent on iron as a cofactor and would require regulation of iron homeostasis [14]. However, the activation of the mTOR pathway is considered to be the driving force in AD pathogenesis. To date, the mechanism through which the downregulation of mTOR signaling triggers the degradation of amyloid-β and prevents the further accumulation of tau protein has been revealed [15]. As a result, targeting the inhibition of mTOR has been widely employed in age-related diseases to slow down the progression of AD [16].

Senescent cells undergo a phenotype development that extends beyond growth inhibition, characterized by irreversible cell cycle arrest with distinct phenotypic alterations and molecular changes within the cells [17]. Common features arising in senescent cells are the enhanced enzymatic activity of the lysosomal hydrolase senescence-associated β-galactosidase (SA-β-gal) and cell cycle checkpoint protein p53, which involve the inhibition of cyclin-dependent kinases p21 (CDKN1A) activated by DNA damage response (DDR) and is one of the major signaling pathway towards mitochondrial dysfunction [17, 18]. It is well established that p53 acts as an upstream regulator of mTOR signaling, which highlights the mechanisms underlying the regulation of iron homeostasis and cellular senescence [19]. One of the most important activities of senescent cells is developing a complex of senescence-associated secretory phenotype (SASP). Its prolonged secretion reinforces the spread of premature senescence to neighboring cells and may contribute to the earlier stage of neurodegenerative disease [20]. In addition, a strong correlation between senescence phenotypes and AD patient brains has been documented in a transcriptomic analysis study [21]. Previous *in vitro* studies have demonstrated that treating cells with ferric ammonium citrate (FAC) can induce cellular senescence due to iron dysregulation [22, 23]. Conversely, these senescent cells exhibit resistance to iron-induced ferroptosis [23]. Therefore, using synthetic iron inhibitor compounds as novel tools in the treatment of cell senescence and highlighting the efficacy of these agents, are the critical steps for neurodegenerative disease interception.

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is a synthetic organoselenium compound first described in redox biology as a potent antioxidant [24]. It is a well-known mimicking glutathione peroxidase (GPx) that degrades hydrogen peroxide (H_2O_2) and organic peroxides in the brain [24]. Ebselen has been studied extensively in clinical trials to limit the neurological deficits that occur in ischemic stroke [25], bipolar disorders [26], and AD [27]. Previous studies reported that ebselen is a potent inhibitor of DMT1-mediated Fe^{2+} uptake, limiting iron-catalyzed cellular damage [28–30]. To date, ebselen has not been widely used as a therapeutic agent for reducing senescent-like neuronal cells caused by iron overload-mediated brain aging.

We speculate that DMT1 iron uptake might be an early target molecule promoting brain iron dyshomeostasis caused by ferric ammonium citrate (FAC). Therefore, the aims of this study were to determine whether ebselen could alleviate and counteract the iron accumulation-induced senescence in SH-SY5Y cells. The findings may potentially provide an insight into the therapeutic effect of ebselen to counteract iron burden and cellular senescence via its antiaging effect.

2. Materials and Methods

2.1. Reagents and Antibodies. Ebselen, ferric ammonium citrate (FAC), and anti-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-DMT1 was obtained from EMD Millipore (Rahway, NJ, USA). Anti-mTORC1 and anti-p-mTORC1 were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-FPN1, anti-p53, and anti-p21 were purchased from Abcam (Cambridge, CB2 0AX, UK). Anti-FT-L and cell culture reagents were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All chemicals used in this study were of analytical grade.

2.2. Cell Culture and Treatments. Human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; CRL-2266). Cells were cultured in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/ streptomycin at $37^{\circ}C$ in a 5% CO_2 humidified incubator. Cells were then cultured until the monolayers reached 80% confluence. The passages from the 3rd to the 6th of cells were used for subsequent experiments.

Once the cells reached approximately 80% confluence, cells were seeded into culture plates. After an overnight serum deprivation, 100 μM FAC in the serum-free medium was applied to the cells for 24 h. Thereafter, the different concentrations of ebselen in serum-free medium (5 and 10 μM) were applied for an additional 24 h. The culture duration for FAC treatment was based on the preliminary time-course experiments, that increased the susceptibility of the expression of iron importer DMT1 protein (see Figures 1(a) and 1(b)).

2.3. Cell Viability Assay.

The cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, SH-SY5Y cells were seeded into 96-well plates at a density of 2×10^4 cells/well. Cells were then treated with 100 μM FAC for 24 h before the addition of the various concentrations of ebselen (0, 3, 5, 10, and 20 μM) and incubated for 24 h. These were followed by the inclusion of 2 mg/ml MTT solution in each well and the 2-hour incubation in an incubator. The MTT solution was then completely removed and dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. The amount of MTT formazan product was measured at 570 nm using the Synergy HT microplate reader (BioTek, Winooski, VT, USA). Cell viability was expressed as a percentage relative to the untreated control.

2.4. Intracellular Calcein-Chelatable Iron Assay.

The levels of the cytosolic labile iron pool (LIP) were estimated using the metal-sensitive fluorophore calcein-AM [31] from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded into 96-well plates at a density of 2×10^4 cells/well. Cells were then treated with 100 μM FAC for 24 h before the addition of ebselen (5 and 10 μM) and incubated for 24 h. Cells were subsequently washed twice with 1X phosphate-buffered saline (PBS) and incubated with 1 μM calcein-AM at 37°C for 30 min. The excessive calcein-AM on the cell surface was gently washed twice with 1X PBS followed by the measurement of the fluorescence intensity using the Synergy HT microplate reader (BioTek, Winooski, VT, USA) at excitation/emission wavelengths of 485/538 nm. The images of the cells were acquired immediately and analyzed by using a fluorescence microscope (ZEISS, Germany).

2.5. Measurement of Intracellular Reactive Oxygen Species (ROS).

The intracellular ROS levels were assessed using the cell-permeable reagent fluorogenic probe, 2′-7′dichlorofluorescin diacetate (DCFDA) from Thermo Fisher Scientific Inc. (Waltham, MA, USA) according to the manufacturer's instructions. Subsequently, the supernatants were completely discarded and the cells were incubated with 1X PBS containing 10 μM DCFDA at 37°C for 30 min. The intensity of 2,7-dichlorofluorescein (DCF) fluorescence was measured using the Synergy HT microplate reader (BioTek, Winooski, VT, USA) at excitation/emission wavelengths of 488/

520 nm. The data were presented as the percentage of ROS relative to the untreated control.

2.6. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$).

The $\Delta\Psi$m was measured using tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) as the indicator of mitochondrial damage (Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, SH-SY5Y cells were seeded into 96-well plates at a density of 2×10^4 cells/well and treated as previously described above. After the treatment, cells were incubated with 5 μM JC-1 at 37°C for 30 min. Cells were then gently washed twice with 1X PBS and the fluorescence intensity was measured using the Synergy HT microplate reader (BioTek, Winooski, VT, USA). The wavelengths of excitation/emission for green-fluorescent monomers were set at 490/530 nm and at 525/590 nm for red fluorescent J-aggregates. The data were presented as the ratio of monomer/J-aggregates.

2.7. Senescence-Associated β-Galactosidase Staining (SA-β-Gal).

The cellular senescence was performed using the senescence β-galactosidase staining kit (Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions. In brief, SH-SY5Y cells were seeded into 24-well plates at a density of 3×10^4 cells/well and treated as previously described above. Cells were washed twice with 1X PBS and fixed in 4% (v/v) formaldehyde for 10 mins at room temperature. This was followed by washing with distilled water for three times and then staining with the β-galactosidase detection solution (pH 6.0) at 37°C in a non-CO$_2$ incubator for 16 h. Cells were observed under an Axiocam 305 color, a phase-contrast microscope (ZEISS, Germany) and the bright blue-staining cells were considered as the β-galactosidase positive cells. Cells were eventually counted for three random vision fields and the calculation of the senescence rate was performed.

2.8. Measurement of Senescence-Associated Secretory Phenotype (SASP).

The SASP was quantified from culture supernatants using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. After the treatment, the particulates were removed by centrifugation at $10,000 \times g$ at 4°C for 10 min and the supernatants were collected and frozen at −80°C until further analysis. The levels of cytokines (IL-6 and IL-8) were assessed using ELISA kits specific to humans (R&D system). The absorbance was measured at 450 nm following the manufacturer's reference using the Synergy HT microplate reader (BioTek, Winooski, VT, USA) and the results were expressed in ng/ml.

2.9. Western Blot Analysis.

Western blot experiments were performed following protocols as described previously [32]. Briefly, SH-SY5Y cells were harvested and subsequently lysed in the radioimmunoprecipitation (RIPA) assay buffer, supplemented with the 1% protease inhibitor cocktail. After centrifugation at $2,000 \times g$ for 15 minutes at 4°C, the protein
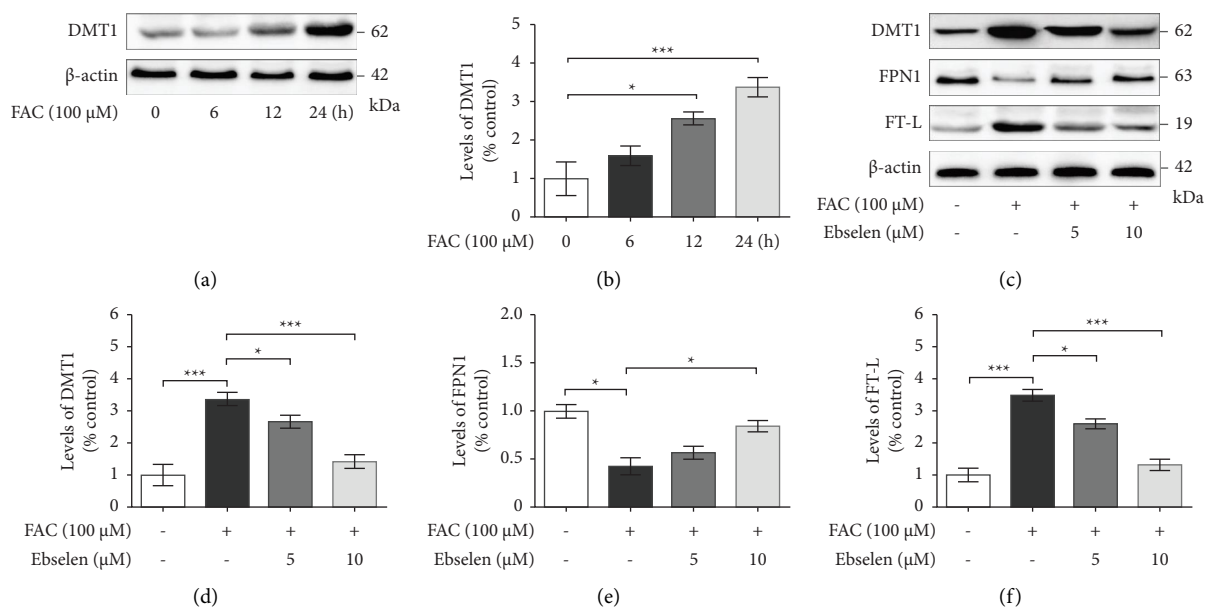
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FIGURE 1: Ebselen attenuated changes in iron regulation following FAC-induced iron overload in SH-SY5Y cells. Cells were exposed to 100 μM FAC for 0, 6, 12, and 24 h. (a) Western blot analysis of the DMT1 in cells exposed to 100 μM FAC at different times and (b) the quantitative analysis of the protein expression normalized using β-actin. Cells were exposed to FAC (100 μM) for 24 h prior to ebselen (5 and 10 μM) for 24 h. (c) Western blot analysis of the DMT1, FPN1, and FT-L, and the quantitative analysis of the (d) DMT1, (e) FPN1, and (f) FT-L protein expression were normalized using β-actin, respectively. The data are the mean ± SEM of the three independent experiments performed in triplicate. $*P < 0.05$ and $***P < 0.001$ vs. the untreated group. $*P < 0.05$ and $***P < 0.001$ vs. the FAC alone group (see supplementary data 1-2 for the original image of Figure 1(a) and supplementary data 3–6 for the original image of Figure 1(c), respectively).

concentration was determined using a bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Equal amounts (60 μg per sample) of proteins per were loaded on 10–15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 100 V for 10 min, followed by 120 V for the remainder of the separation. The gel was transferred onto a 0.22 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and run at 100 V for 90 min. After blocking the nonspecific binding site with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature, the membranes were probed with antibodies against DMT1 (dilution, 1 : 500), FPN1 (dilution, 1 : 1000), FT-L (dilution, 1 : 1000), p53 (dilution, 1 : 2000), p21 (dilution, 1 : 1000), mTORC1 (dilution, 1 : 2000), p-mTORC1 (dilution, 1 : 2000), and β-actin (dilution, 1 : 5000) overnight at 4°C, along with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. The protein bands were enhanced using the SuperSignal West Pico chemiluminescence detection kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and visualized using the ChemiDoc XRS + System. The blots were analyzed using Image Lab Software (Bio-Rad, Hercules, CA, USA) and normalized to the expression of β-actin.

2.10. Statistical Analysis. All values are presented as mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicate. Comparison between groups was analyzed by using one-way ANOVA with Bonferroni's tests. Differences were considered statistically significant at $P < 0.05$. All statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Ebselen Improves SH-SY5Y Cell Viability against FAC-Induced Cytotoxicity. To determine whether the effective concentration of ebselen against the FAC-induced cytotoxicity and the viability of SH-SY5Y cells exposed solely to various concentrations of ebselen (3, 5, 10, and 20 μM) were measured by the MTT assay. The viability of the cells treated with ebselen alone compared to the untreated control cells significantly decreased at the concentration of 20 μM and promoted cell proliferation at the lowest concentration (3 μM of ebselen) (Figure 2(a)). Therefore, the viability effect of 5 μM and 10 μM ebselen treatments were further investigated after the exposure to the 100 μM FAC for 24 h (Figure 2(b)). The results showed that ebselen significantly improved the reduction of cell viability compared with the FAC-treated cells (Figure 2(c)). This highlighted the importance of ebselen in reducing SH-SY5Y cells' toxicity against FAC-induced cytotoxicity. Subsequently, the effective concentrations of FAC (100 μM) and ebselen (5 and 10 μM) were used in all experiments.

3.2. Downregulation of DMT1 Expression by Ebselen Enhances the Proper Iron Homeostasis in FAC-Treated SH-SY5Y Cells. The expression of iron uptake-mediated DMT1 in response to the varied exposure times of 100 μM
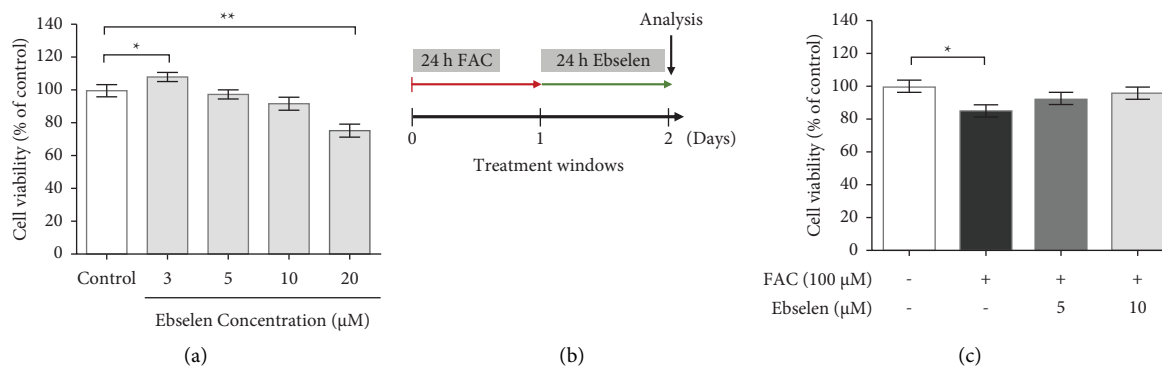
FIGURE 2: Ebselen improved viability in SH-SY5Y after the exposure to FAC. The cell viability was determined using the MTT assay. (a) Cell viability after the exposure to the serum-free medium containing various concentrations of ebselen for 24 h. (b) Ebselen treatment paradigm following FAC-induced intracellular iron accumulation. (c) Cell viability after the exposure to FAC (100 μM) prior ebselen (5 and 10 μM). The data are the mean ± SEM of the three independent experiments performed in triplicate. $^*P < 0.05$ and $^{**}P < 0.01 vs.$ the untreated group.

FAC (0, 6, 12, and 24 h) was determined in SH-SY5Y cells. The results showed that the cells treated with FAC for 24 h markedly upregulated DMT1 protein expression compared to the untreated control cells (Figures 1(a) and 1(b)) (see Supplementary data 1-2 for the original image of Figure 1(a)). Therefore, this exposure time (24 h) was selected for the examination of the effect of ebselen in the SH-SY5Y cells after 100 μM FAC treatment. The results revealed that ebselen significantly decreased FAC-induced iron influx into the cell through DMT1 compared with that of the FAC-treated group (Figures 1(c) and 1(d)). The impact of ebselen on the key components in iron homeostasis in FAC-treated SH-SY5Y cells was also evaluated. FPN1, the only mammalian iron exporter identified to date [33] and FT-L, the major protein responsible for intracellular iron storage, were measured using Western blot analyses (see Supplementary data 3–6 for the original image of Figure 1(c)). FAC alone significantly reduced FPN1, but increased FT-L protein expressions compared with that of the untreated control cells. This was rescued following the ebselen treatment (Figures 1(b), 1(e), and 1(f)) which attenuated changes in the iron regulation. Moreover, ebselen also inhibits iron influx that may accelerate the extracellular iron transport.

3.3. Ebselen Reduces the Intracellular LIP Level in FAC-Induced Iron Overload in SH-SY5Y Cells.
The potential effects of ebselen on the cytosolic LIP level in FAC-treated SH-SY5Y cells were evaluated using the calcein-AM assay; and the reduction in calcein-AM fluorescence intensity signifies an increase in the cytosolic-chelatable Fe^{2+} [34]. The patches of green fluorescence were notably increased in the cytosol and diffused into lysosomes, where they were cleaved by the lysosomal esterase resulting in the calcein-AM penetration (Figure 3(a)). Therefore, the results indicated that the cytosolic LIP level significantly decreased following the ebselen treatment. However, this was rescued by the ebselen treatment (Figure 3(b)). These findings suggest that ebselen could directly inhibit iron influx and control the levels of cytosolic LIP in cultured SH-SY5Y cells.

3.4. Ebselen Suppresses ROS Accumulation and Rescues the Loss of $\Delta\Psi m$ by FAC-Induced Iron Overload in SH-SY5Y Cells.
It is well documented that excessive iron is involved in an imbalance of redox homeostasis, resulting in the increase of intracellular ROS levels and oxidative stress [35]. This research investigated the antioxidative property of ebselen against FAC-induced intracellular ROS formation in SH-SY5Y cells using a DCFH-DA probe. The treatment with FAC alone significantly increased the intracellular ROS level compared with the untreated control cells. Interestingly, this was significantly decreased by ebselen treatment and the level was comparable to that in the control group (Figure 3(c)). These findings suggest that ebselen possesses antioxidant properties, which could potentially dampen the depolarization of mitochondrial membrane potential ($\Delta\Psi M$) of aging neuronal cells caused by oxidative stress. The potential effects of ebselen on the rescuing of the oxidative stress-induced $\Delta\Psi m$ impairment were confirmed using the JC-1 probe. The results revealed a significant decrease in the ratio of green/red fluorescence intensity in the FAC-treated cells, which implied the impairment of $\Delta\Psi M$. This was rescued by the ebselen treatment, resulting in the increase of the green/red fluorescence intensity ratio (Figure 3(d)).

3.5. Ebselen Restores SH-SY5Y Senescence via Inhibiting the mTORC1 Signaling Pathway.
The potential mechanisms of ebselen in restoring the iron overload-associated senescence and oxidative stress in SH-SY5Y cells were evaluated using a collection of cellular senescence markers including SA-β-gal, SASP, and cell cycle inhibitors activated by DNA damage response (DDR). This is due to the fact that senescent feature alone is insufficient to determine a senescent state. The iron overload in the SH-SY5Y cells is firmly associated with an increased number of SA-β-gal positive cells compared with the untreated control cells, which were almost unaffected. In contrast, the SA-β-gal positive cells were partially reverted following the ebselen treatment, particularly at a concentration of 10 μM (Figures 4(a) and 4(b)). Since SASP has been shown to induce paracrine
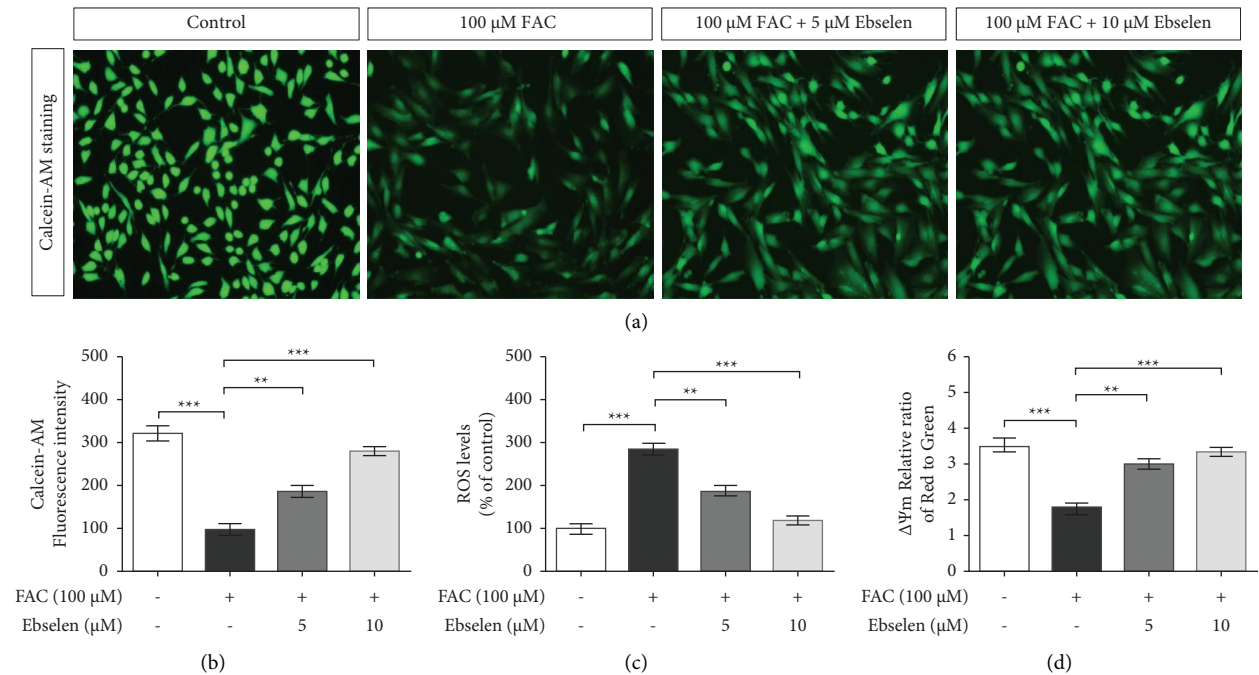
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FIGURE 3: Ebselen reduced the intracellular iron level and oxidative stress following FAC-induced iron overload in SH-SY5Y cells. Cells were exposed to FAC (100 μM) for 24 h prior to ebselen (5 and 10 μM) for 24 h. The intracellular iron level based on the redox-active cytosolic LIP was examined using calcein-AM. (a) Representative images of cytosolic LIP were observed by using fluorescence microscope and taken at ×200 magnification. (b) The quantitative analysis of the intracellular iron level and the decreased calcein-AM fluorescence intensity indicate an increased intracellular iron level. (c) The quantitative analysis of the intracellular ROS level using DCFH-DA probe. (d) The quantitative analysis of $\Delta\Psi$m using JC-1 dye. The data are the mean ± SEM of four independent experiments performed in triplicate. $^{***}P < 0.001$ vs. the untreated group. $^{**}P < 0.01$ and $^{***}P < 0.001$vs. the FAC alone group.
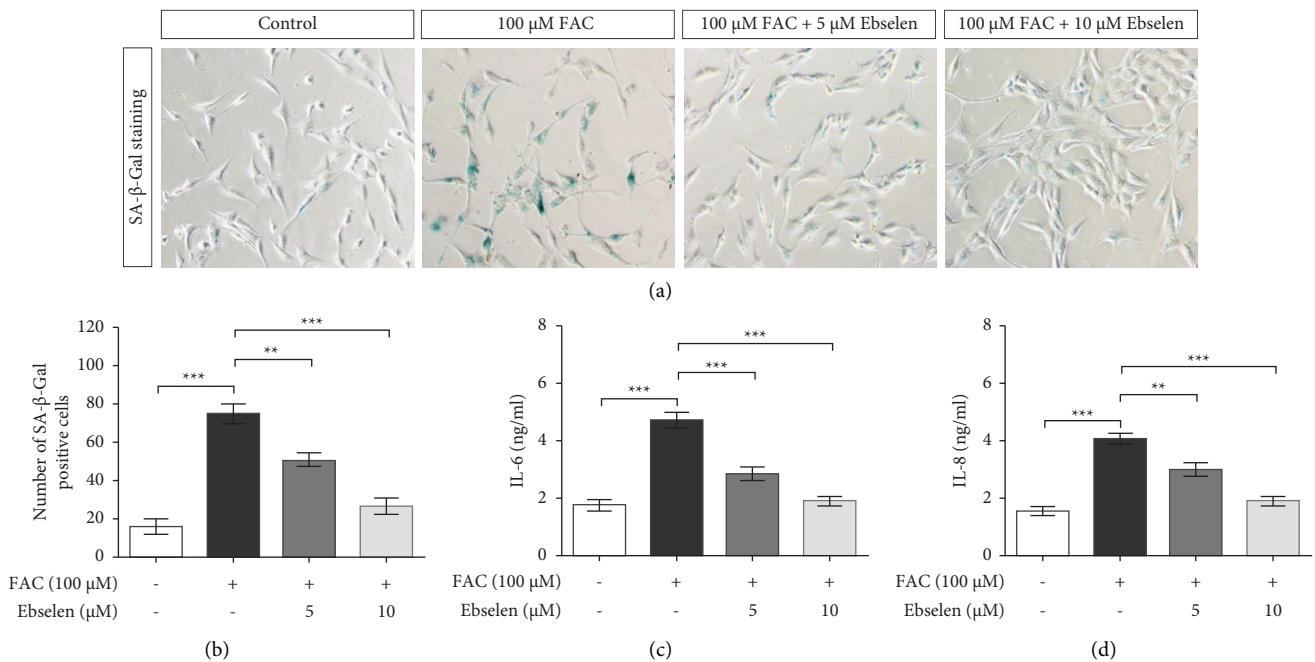

(a)

(b)

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FIGURE 4: Ebselen rescued iron overload-induced neuronal senescence. Cells were exposed to FAC (100 μM) for 24 h prior to ebselen (5 and 10 μM) for 24 h and then the senescence markers were measured. (a) Representative images of SH-SY5Y stained with SA-β-gal, the images were obtained by phase-contrast microscopy, and taken at ×200 magnification. (b) The percentage of SA-β-gal-positive cells per field and three vision fields from each replica were scored. The amount of SASP secretion, (c) IL-6, and (d) IL-8, was analyzed using ELISA. The data are the mean ± SEM of the three independent experiments performed in triplicate. $^{***}P < 0.001$ vs. the untreated group. $^{**}P < 0.01$ and $^{***}P < 0.001$vs. the FAC alone group.

senescence and chronic inflammation in the aging brain, the expression of a common SASP component, including IL-6 and IL-8, using ELISA was performed. The results revealed that there was a marked increase in IL-6 and IL-8 proteins in the senescent SH-SY5Y culture media, which was significantly reduced after the ebselen treatment (Figures 4(c) and 4(d)). Furthermore, the iron overload and the overproduction of ROS increased the activation of p53 and the expression of downstream proteins p21, which were effectively reversed by ebselen (Figures 5(a)–5(c)) (see Supplementary data 7–9 for the original image of Figure 5(a)). The investigation of mTORC1 activity was also carried out and the results showed that the oxidative stress induced the phosphorylation of mTORC1. Conversely, ebselen significantly downregulated the phosphorylation of mTORC1 (Figures 5(d)–5(f)), demonstrating that iron overload creates neuronal senescence through the activation of the mTORC1 signaling pathway (see Supplementary data 10–12 for the original image of Figure 5(d)).

4. Discussion

Iron accumulation is the function of aging as it plays a pivotal role in activating a cascade of pathophysiological processes in cellular senescence via oxidative stress. Ebselen, a restoring antiaging agent, was investigated in the present study to determine its restoring potential in the iron overload-induced senescence-like neuronal cells, SH-SY5Y.

Iron overload in the brain amplifies neuronal toxicity through its redox-active effects, leading to cellular senescence, which preferentially occurs in physiological brain aging and corresponds to the severity of neurodegenerative diseases, especially AD and PD lesions [36, 37]. Given the last decade of clinical trials, drug therapeutic for AD has largely been directed at the Aβ peptide, owing to the hypothesis of disease that it is a critical initiator of the progression of lesions [38]. However, medication to modify the pathogenesis of AD has not yet been identified. Ebselen, on the other hand, is a widely recognized synthetic peroxynitrite scavenger compound that mimics the activity of GPx, which catalyzes essential reactions that provide protection against oxidative damage [24]. Ebselen has been widely used as a therapeutic agent in the clinical trials of various diseases [39, 40]. A recent study demonstrated that ebselen acts as a potent anti-inflammatory agent by suppressing the TRAF2-ASK1-SEK1 signaling pathway, which is essential for endoplasmic reticulum stress-induced cell death [41], and is consistent with the fact that ebselen has neuropharmacological properties, which possess antioxidant and anti-inflammatory activity and free radicals scavenging [42]. In addition, dyshomeostasis of brain iron causes aggregation of Aβ peptides and tau protein, consequently promoting neuronal cytotoxicity, which is a pathological hallmark of AD [43, 44]. Several studies have revealed the potential effects of ebselen in preventing the progression of AD-related pathology and enhancing the spatial learning and memory in two-month-old $3 \times$ Tg-AD mice, suggesting that ebselen could be a potential therapeutic agent for the prevention or treatment of AD [45]. Furthermore, ebselen also has multifunctional properties, including reversing oxidative stress, neuronal apoptosis, and memory impairment in a sporadic model of AD induced by intracerebroventricular administration of streptozocin [27]. These findings imply that ebselen could be a therapeutic target for AD. Iron has also been shown to enhance ferroptosis, a type of regulated necrosis characterized by iron-induced lipid peroxidation that causes catastrophic membrane rupture, which is controlled by GPx4, an event that occurs in AD progression [46]. Due to the GPX mimetic properties, the selenium compound ebselen could reduce neuronal ferroptosis in traumatic brain injury with cerebral ischemia and intracerebral hemorrhage, respectively [47, 48]. Nonetheless, there are few studies elucidating the benefits of ebselen as a treatment strategy for curing brain iron overload-associated senescence-like neuronal cells that accelerate the development of AD.

The mechanism underlying iron progressively accumulating in senescent cells has not been fully understood. We first examined the potential of ebselen to alleviate the dysregulation of iron homeostasis in SH-SY5Y cells following the exposure to FAC. Ebselen has been identified as a DMT1 inhibitor, which plays a vital role in direct DMT1 transport blockage and iron-catalyzed damage limitation [28]. This could reverse the dopaminergic neuronal loss and motoric deficit in the brain regions implicated in PD [49]. Neuronal iron transport is principally dependent on the NTBI transport pathway via DMT1 [4]. Our results demonstrated that ebselen significantly decreased DMT1 expression upon the increase of intracellular iron level, indicating that the high-affinity property of iron chelator inhibits iron uptake and pumps iron into the cytosol [31, 50]. In addition, FAC-induced dysregulation of iron homeostasis was considerably mitigated by ebselen. Since ebselen increased FPN1 expression, while decreasing the expression of FT-L and the cytosolic LIP levels; it might exert critical routes to DMT1 inhibitor-mediated FPN1 degradation that plays an essential role in the export of iron. Possibly, ebselen may chelate labile iron to form a relatively stable compound to maintain iron homeostasis [29, 30].

The collective evidence shows that Fe^{2+} is an effective modulator to catalyze the conversion of H_2O_2 to hydroxyl radicals via the Fenton reaction [5]. This is in line with many studies which have reported that increased LIP leads to a marked increase in ROS formation, the key propagation to DNA damage and mitochondrial function decline observed in mammalian aging [51, 52]. Our results showed that ebselen markedly decreased intracellular ROS levels and increased $\Delta\Psi m$ following the iron overload in SH-SY5Y cells. These outcomes provide a strong evidence supporting the direct property of ebselen as a powerful antioxidant and radical scavenger in the ischemia/reperfusion model [25]. As a consequence of the extensive oxidative injury, cells could either react toward a defensive mechanism or the pathological process, leading to mitochondrial dysfunction. This is apparently distinct in the accumulation of senescent cells and secretory phenotype [53, 54].

Despite being postmitotic cells, neurons could be capable of undergoing senescence in response to oxidative stress [55]. Further experiments must be carried out to evaluate
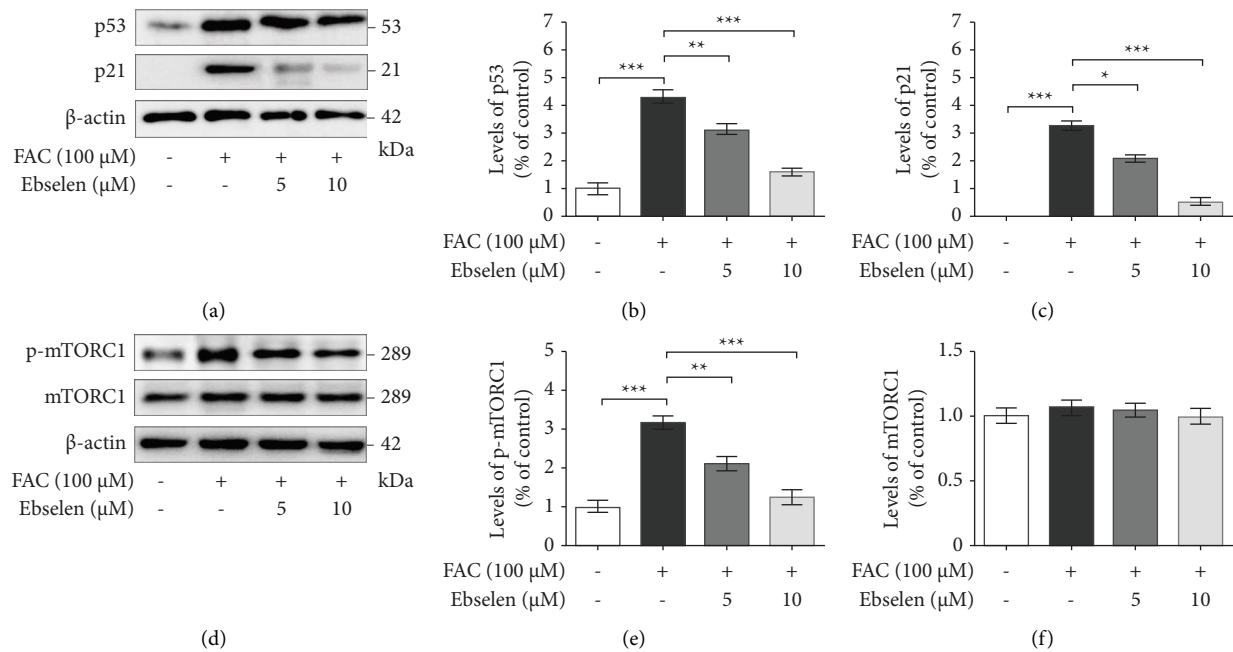
(a)

(b)

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(e)

(f)

Figure 5: Ebselen counteracts neuronal senescence through the mTORC1 pathway. Cells were exposed to FAC (100 μM) for 24 h prior to ebselen (5 and 10 μM) for 24 h and then the expression of markers for the cell cycle was measured. (a) Cell cycle checkpoint protein p53/ cyclin-dependent kinase (CDK) inhibitors p21 were evaluated using the Western blot analysis. The quantitative analysis of the expression of (b) p53 and (c) p21 was normalized using β-actin, respectively. (d) Western blot analysis of phosphorylated p-mTORC1 and total mTORC1. The quantitative analysis of the expression of (e) p-mTORC1 and (f) total mTORC1 was normalized using β-actin, respectively. The data are the mean ± SEM of the three independent experiments performed in triplicate. ***$P < 0.001$ vs. the untreated group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. the FAC alone group (see supplementary data 7–9 for the original image of Figure 5(a) and supplementary data 10–12 for the original image of Figure 5(d), respectively).

whether iron accumulation might be a critical factor in brain aging and to assess the property of ebselen on antiaging in reliable neuronal senescence. The molecular mechanism responsible for cellular senescence demonstrated the downregulation of the nuclear receptor coactivator 4 (NCOA4), a selective cargo receptor for autophagic turnover of ferritin, which induces ferritin accumulation and increases DNA instability and lysosomal dysfunction [56, 57]. Consequently, senescent cells likely accumulate iron partially due to failure to degrade ferritin [58], thereby sequestering iron in an inactive state and leading to cell-cycle arrest [23]. Besides, SA-β-Gal is the most widely used marker of senescence derived from lysosomal enzymes and can be detected at early stages. It is well established that excessive ROS strongly triggers cellular senescence via regulating multiple pathways. Proinflammatory cytokines, termed senescence-associated secretory phenotype (SASP), can increase c-Jun N-terminal kinase (JNK) phosphorylation [59] and activate the ROS/NF-κB pathway [30], thus accelerating cellular senescence. The SASP paracrine signaling is a crucial pathological feature of cellular senescence that causes local chronic inflammation [18, 60]. Not only glial cells are responsible for releasing proinflammatory cytokines, but also neurons, resulting in a self-propelling cycle of neuroinflammation and eventually neurodegeneration [61]. Our results demonstrated that ebselen could restore neuronal

senescence by significantly reducing β-gal activity and decreasing the expression of IL-6 and IL-8 levels, which is related to its potent anti-inflammatory properties [62].

In addition, the appearance of senescence phenotype in our study demonstrated that neurons respond to the damage by activating the p53 pathway and its downstream, p21, known as CDKN1A (cyclin-dependent kinase inhibitor 1) [17, 18]. Phosphorylation of p53 has been shown to be critical for telomeric stress-induced cellular senescence and is closely linked to DNA double-stranded breaks (DSBs) and DNA damage response (DDR) activation [63]. Ebselen notably restored neuronal senescence by the downregulation of p53/ p21 expression. The results provide evidence that ebselen could decrease the acceleration of senescence through the reduction of ROS levels. By simultaneously investigating the signaling pathway involved in cellular senescence in parallel experiments, we found that ebselen could induce the downregulation of mTORC1 expression, a key mediator of cellular senescence. It has been reported that oxidative stress-dependent mitochondrial depletion exacerbates the proaging phenotype thereby promoting the mTOR phosphorylation cascade [64]. Therefore, the inhibition of the mTORC1 pathway by ebselen might restore iron overload-induced senescence-like neuronal cells, SH-SY5Y, by suppressing the SASP regulatory proteins, thereby improving the mitochondrial integrity and reversing cell cycle arrest.
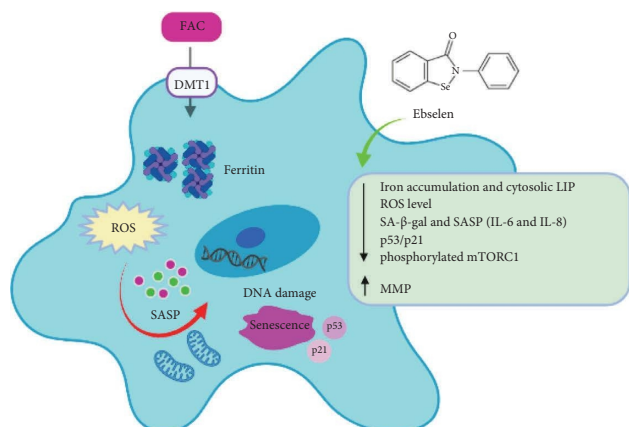
FIGURE 6: A schematic model representation of the proposed mechanism of ebselen on FAC-induced iron overload associated with senescent-like neuronal cells, SH-SY5Y. FAC facilitates an increased intracellular iron levels via DMT1, the main pathway responsible for cellular iron uptake in neurons. Subsequently, increasing intracellular ROS formation via the Fenton reaction that mediates proinflammatory cytokine release leads to the damage of mitochondrial membrane and DNA. Excessive intracellular iron levels are undoubtedly one of the main causes of cellular senescence, leading to organismal aging. Ebselen is a DMT1 inhibitor that could decrease intracellular iron levels by increasing the expression of FPN1 iron exporter and decreasing FT-L iron storage. Furthermore, ebselen could rescue neuronal senescence by reducing ROS levels and by improving $\Delta\Psi$m and cellular senescence markers, including SA-β-gal, SASP profile, and p53/p21 via inhibiting the mTORC1 signaling pathway.

5. Conclusions

We conclude that excessive iron plays a pivotal role in accelerating the processes of brain aging, especially neurodegenerative diseases. Our study provides evidence that ebselen, a DMT1 inhibitor, might be one of the therapeutic options for mitigating iron overload-associated senescence-like neuronal cells, SH-SY5Y, through direct and indirect antioxidative and anti-inflammatory activities (Figure 6). While we find that these studies offer significant insights that contribute to the scientific community's ongoing interest, it remains crucial to recognize that our current work is merely the initial step. The efficacy of ebselen appears to prompt the need for subsequent *in vivo* studies, which we anticipate will be undertaken in the near future. These studies will likely pave the way for eventual clinical trials, constituting a critical phase in uncovering the full potential of ebselen as a prospective neurodegenerative medicine. In our perspective, our present research and the forthcoming studies will collectively advance our understanding of ebselen's therapeutic value and contribute to addressing the challenges posed by neurodegenerative diseases. The combination of *in vivo* investigations and clinical trials will provide deeper insights into ebselen's efficacy and its potential impact as a future therapeutic medicine.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Supplementary Materials

Supplementary data 1: original image of divalent metal transporter 1 (DMT1) blot for Figure 1(a). Supplementary data 2: original image of β-actin blot for Figure 1(a). Supplementary data 3: original image of divalent metal transporter 1 (DMT1) blot for Figure 1(c). Supplementary data 4: original image of ferroportin 1 (FPN1) blot for Figure 1(c). Supplementary data 5: original image of ferritin light chain (FT-L) blot for Figure 1(c). Supplementary data 6: original image of β-actin blot for Figure 1(c). Supplementary data 7: original image of p53 blot for Figure 5(a). Supplementary data 8: original image of p21 blot for Figure 5(a). Supplementary data 9: original image of β-actin blot for Figure 5(a). Supplementary data 10: original image of p-mTORC1 blot for Figure 5(d). Supplementary data 11: original image of mTORC1 blot for Figure 5(d). Supplementary data 12: original image of β-actin blot for Figure 5(d). (*Supplementary Materials*)

References

[1] R. J. Ward, F. A. Zucca, J. H. Duyn, R. R. Crichton, and L. Zecca, "The role of iron in brain ageing and neurodegenerative disorders," *The Lancet Neurology*, vol. 13, no. 10, pp. 1045–1060, 2014.

[2] R. C. McCarthy and D. J. Kosman, "Iron transport across the blood-brain barrier: development, neurovascular regulation and cerebral amyloid angiopathy," *Cellular and Molecular Life Sciences*, vol. 72, no. 4, pp. 709–727, 2015.

[3] A. I. Khan, J. Liu, and P. Dutta, "Iron transport kinetics through blood-brain barrier endothelial cells," *Biochimica et Biophysica Acta (BBA)-General Subjects*, vol. 1862, no. 5, pp. 1168–1179, 2018.

[4] T. Skjørringe, A. Burkhart, K. B. Johnsen, and T. Moos, "Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology," *Frontiers in Molecular Neuroscience*, vol. 8, p. 19, 2015.

[5] A. Carocci, A. Catalano, M. S. Sinicropi, and G. Genchi, "Oxidative stress and neurodegeneration: the involvement of iron," *Biometals*, vol. 31, no. 5, pp. 715–735, 2018.

[6] R. Cheng, V. V. Dhorajia, J. Kim, and Y. Kim, "Mitochondrial iron metabolism and neurodegenerative diseases," *Neurotoxicology*, vol. 88, pp. 88–101, 2022.

[7] J. R Connor and A. J. Ghio, "The impact of host iron homeostasis on disease," *Biochimica et Biophysica Acta (BBA)-General Subjects*, vol. 1790, no. 7, pp. 581-582, 2009.

[8] G. Gao, L. You, J. Zhang, Y. Z. Chang, and P. Yu, "Brain iron metabolism, redox balance and neurological diseases," *Antioxidants*, vol. 12, no. 6, p. 1289, 2023.

[9] M. Núñez, D. Borquez, and P. Urrutia, "Iron, the endolysosomal system and neuroinflammation: a matter of balance," *Neural Regeneration Research*, vol. 17, no. 5, pp. 1003-1004, 2022.

[10] R. A. Saxton and D. M. Sabatini, "mTOR signaling in growth, metabolism, and disease," *Cell*, vol. 169, no. 2, pp. 361–371, 2017.

[11] M. A. Shafei, M. Harris, and M. E. Conway, "Divergent metabolic regulation of autophagy and mTORC1-early events in Alzheimer's disease?" *Frontiers in Aging Neuroscience*, vol. 9, p. 173, 2017.

[12] D. Papadopoli, K. Boulay, L. Kazak et al., "mTOR as a central regulator of lifespan and aging," *F1000Research*, vol. 8, 2019.

[13] T. Weichhart, "mTOR as regulator of lifespan, aging, and cellular senescence: a mini-review," *Gerontology*, vol. 64, no. 2, pp. 127–134, 2018.

[14] C. Shang, H. Zhou, W. Liu, T. Shen, Y. Luo, and S. Huang, "Iron chelation inhibits mTORC1 signaling involving activation of AMPK and REDD1/Bnip3 pathways," *Oncogene*, vol. 39, no. 29, pp. 5201–5213, 2020.

[15] A. Subramanian, T. Tamilanban, A. Alsayari et al., "Trilateral association of autophagy, mTOR and Alzheimer's disease: potential pathway in the development for Alzheimer's disease therapy," *Frontiers in Pharmacology*, vol. 13, Article ID 1094351, 2022.

[16] J. B. Mannick and D. W. Lamming, "Targeting the biology of aging with mTOR inhibitors," *Nature Aging*, vol. 3, no. 6, pp. 642–660, 2023.

[17] R. Kumari and P. Jat, "Mechanisms of cellular senescence: cell cycle arrest and senescence associated secretory phenotype," *Frontiers in Cell and Developmental Biology*, vol. 9, no. 6, Article ID 645593, 2021.

[18] V. Gorgoulis, P. D. Adams, A. Alimonti et al., "Cellular senescence: defining a path forward," *Cell*, vol. 179, no. 4, pp. 813–827, 2019.

[19] Y. Liu and W. Gu, "The complexity of p53-mediated metabolic regulation in tumor suppression," *Seminars in Cancer Biology*, vol. 85, pp. 4–32, 2022.

[20] L. Á. Maciel-Barón, S. L. Morales-Rosales, A. Silva-Palacios et al., "The secretory phenotype of senescent astrocytes isolated from Wistar newborn rats changes with anti-inflammatory drugs, but does not have a short-term effect on neuronal mitochondrial potential," *Biogerontology*, vol. 19, no. 5, pp. 415–433, 2018.

[21] E. P. Crowe, F. Tuzer, B. D. Gregory et al., "Changes in the transcriptome of human astrocytes accompanying oxidative stress-induced senescence," *Frontiers in Aging Neuroscience*, vol. 8, p. 208, 2016.

[22] F. Yang, G. Yan, Y. Li et al., "Astragalus polysaccharide attenuated iron overload-induced dysfunction of mesenchymal stem cells via suppressing mitochondrial ROS," *Cellular Physiology and Biochemistry*, vol. 39, no. 4, pp. 1369–1379, 2016.

[23] S. Masaldan, S. A. S. Clatworthy, C. Gamell et al., "Iron accumulation in senescent cells is coupled with impaired ferritinophagy and inhibition of ferroptosis," *Redox Biology*, vol. 14, pp. 100–115, 2018.

[24] A. Müller, E. Cadenas, P. Graf, and H. Sies, "A novel biologically active seleno-organic compound—1," *Biochemical Pharmacology*, vol. 33, no. 20, pp. 3235–3239, 1984.

[25] J. Y. Seo, C. H. Lee, J. H. Cho et al., "Neuroprotection of ebselen against ischemia/reperfusion injury involves GABA shunt enzymes," *Journal of the Neurological Sciences*, vol. 285, no. 1-2, pp. 88–94, 2009.

[26] N. Singh, A. L. Sharpley, U. E. Emir et al., "Effect of the putative lithium mimetic ebselen on brain myo-inositol, sleep, and emotional processing in humans," *Neuropsychopharmacology*, vol. 41, no. 7, pp. 1768–1778, 2016.

[27] F. Martini, S. G. Rosa, I. P. Klann et al., "A multifunctional compound ebselen reverses memory impairment, apoptosis and oxidative stress in a mouse model of sporadic Alzheimer's disease," *Journal of Psychiatric Research*, vol. 109, pp. 107–117, 2019.

[28] H. A. Wetli, P. D. Buckett, and M. Wessling-Resnick, "Small-molecule screening identifies the selanazal drug ebselen as a potent inhibitor of DMT1-mediated iron uptake," *Chemistry & Biology*, vol. 13, no. 9, pp. 965–972, 2006.

[29] L. Xie, W. Zheng, N. Xin, J. W. Xie, T. Wang, and Z. Y. Wang, "Ebselen inhibits iron-induced tau phosphorylation by attenuating DMT1 up-regulation and cellular iron uptake," *Neurochemistry International*, vol. 61, no. 3, pp. 334–340, 2012.

[30] L. Xie, D. Yu, J. Hu et al., "DMT1 inhibitor ebselen inhibits iron-induced amyloidogenic APP processing," *International Journal of Clinical and Experimental Medicine*, vol. 11, no. 8, pp. 7907–7916, 2018.

[31] S. Epsztejn, O. Kakhlon, H. Glickstein, W. Breuer, and Z. Cabantchik, "Fluorescence analysis of the labile iron pool of mammalian cells," *Analytical Biochemistry*, vol. 248, no. 1, pp. 31–40, 1997.

[32] S. Mukem, T. Thongbuakaew, and K. Khornchatri, "Mito-Tempo suppresses autophagic flux via the PI3K/Akt/mTOR signaling pathway in neuroblastoma SH-SY5Y cells," *Heliyon*, vol. 7, no. 6, Article ID e07310, 2021.

[33] Q. Wu, Q. Hao, H. Li et al., "Brain iron deficiency and affected contextual fear memory in mice with conditional Ferroportin1 ablation in the brain," *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, vol. 35, no. 2, Article ID e21174, 2021.

[34] M. Tenopoulou, T. Kurz, P. T. Doulias, D. Galaris, and U. T. Brunk, "Does the calcein-AM method assay the total cellular 'labile iron pool' or only a fraction of it?" *Biochemical Journal*, vol. 403, no. 2, pp. 261–266, 2007.

[35] L. B. Li, R. Chai, S. Zhang et al., "Iron exposure and the cellular mechanisms linked to neuron degeneration in adult mice," *Cells*, vol. 8, no. 2, p. 198, 2019.

[36] C. C. Walton and J. K. Andersen, "Unknown fates of (brain) oxidation or UFO: close encounters with neuronal senescence," *Free Radical Biology and Medicine*, vol. 134, pp. 695–701, 2019.

[37] T. Sato, J. S. Shapiro, H. C. Chang, R. A. Miller, and H. Ardehali, "Aging is associated with increased brain iron through cortex-derived hepcidin expression," *Elife*, vol. 11, Article ID e73456, 2022.

[38] S. Nikseresht, A. I. Bush, and S. Ayton, "Treating Alzheimer's disease by targeting iron," *British Journal of Pharmacology*, vol. 176, no. 18, pp. 3622–3635, 2019.

[39] K. Suzuki, "Anti-oxidants for therapeutic use: why are only a few drugs in clinical use?" *Advanced Drug Delivery Reviews*, vol. 61, no. 4, pp. 287–289, 2009.

[40] C. Santi, C. Scimmi, and L. Sancineto, "Ebselen and analogues: pharmacological properties and synthetic strategies for their preparation," *Molecules*, vol. 26, no. 14, p. 4230, 2021.

[41] Q. Lu, Y. Cai, C. Xiang et al., "Ebselen, a multi-target compound: its effects on biological processes and diseases," *Expert Reviews in Molecular Medicine*, vol. 23, p. E12, 2021.

[42] J. Wang, P. Wang, C. Dong et al., "Mechanisms of ebselen as a therapeutic and its pharmacology applications," *Future Medicinal Chemistry*, vol. 12, no. 23, pp. 2141–2160, 2020.

[43] M. Bulk, B. Kenkhuis, L. M. van der Graaf, J. J. Goeman, R. Natté, and L. van der Weerd, "Postmortem T 2∗- weighted MRI imaging of cortical iron reflects severity of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 65, no. 4, pp. 1125–1137, 2018.

[44] A. Gleason and A. I. Bush, "Iron and ferroptosis as therapeutic targets in Alzheimer's disease," *Neurotherapeutics*, vol. 18, no. 1, pp. 252–264, 2021.

[45] Y. Xie, Y. Tan, Y. Zheng, X. Du, and Q. Liu, "Ebselen ameliorates β-amyloid pathology, tau pathology, and cognitive impairment in triple-transgenic Alzheimer's disease mice," *JBIC, Journal of Biological Inorganic Chemistry*, vol. 22, no. 6, pp. 851–865, 2017.

[46] D. Zhao, K. Yang, H. Guo et al., "Mechanisms of ferroptosis in Alzheimer's disease and therapeutic effects of natural plant products: a review," *Biomedicine & Pharmacotherapy*, vol. 164, Article ID 114312, 2023.

[47] Q. Z. Tuo, S. Masaldan, A. Southon et al., "Characterization of selenium compounds for anti-ferroptotic activity in neuronal cells and after cerebral ischemia–reperfusion injury," *Neurotherapeutics*, vol. 18, no. 4, pp. 2682–2691, 2021.

[48] S. Ren, Y. Chen, L. Wang, and G. Wu, "Neuronal ferroptosis after intracerebral hemorrhage," *Frontiers in Molecular Biosciences*, vol. 9, Article ID 966478, 2022.

[49] C. Liu, C. W. Zhang, S. Q. Lo et al., "S-nitrosylation of divalent metal transporter 1 enhances iron uptake to mediate loss of dopaminergic neurons and motoric deficit," *Journal of Neuroscience*, vol. 38, no. 39, pp. 8364–8377, 2018.

[50] J. W. Rhee, H. Yi, D. Thomas et al., "Modeling secondary iron overload cardiomyopathy with human induced pluripotent stem cell-derived cardiomyocytes," *Cell Reports*, vol. 32, no. 2, Article ID 107886, 2020.

[51] A. Picca, S. K. Saini, R. T. Mankowski et al., "Altered expression of mitoferrin and frataxin, larger labile iron pool and greater mitochondrial DNA damage in the skeletal muscle of older adults," *Cells*, vol. 9, no. 12, p. 2579, 2020.

[52] S. K. Ghosh-Choudhary, J. Liu, and T. Finkel, "The role of mitochondria in cellular senescence," *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, vol. 35, no. 12, Article ID e21991, 2021.

[53] A. Cozzi, D. I. Orellana, P. Santambrogio et al., "Stem cell modeling of neuroferritinopathy reveals iron as a determinant of senescence and ferroptosis during neuronal aging," *Stem Cell Reports*, vol. 13, no. 5, pp. 832–846, 2019.

[54] S. Miwa, S. Kashyap, E. Chini, and T. von Zglinicki, "Mitochondrial dysfunction in cell senescence and aging," *The Journal of Clinical Investigation*, vol. 132, no. 13, Article ID e158447, 2022.

[55] D. Jurk, C. Wang, S. Miwa et al., "Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response," *Aging Cell*, vol. 11, no. 6, pp. 996–1004, 2012.

[56] J. D. Mancias, X. Wang, S. P. Gygi, J. W. Harper, and A. C. Kimmelman, "Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy," *Nature*, vol. 509, no. 7498, pp. 105–109, 2014.

[57] J. D. Mancias, L. Pontano Vaites, S. Nissim et al., "Ferritinophagy via NCOA4 is required for erythropoiesis and is regulated by iron dependent HERC2-mediated proteolysis," *Elife*, vol. 4, Article ID e10308, 2015.

[58] C. D. Wiley and J. Campisi, "The metabolic roots of senescence: mechanisms and opportunities for intervention," *Nature Metabolism*, vol. 3, no. 10, pp. 1290–1301, 2021.

[59] R. Yarza, S. Vela, M. Solas, and M. J. Ramirez, "c-Jun N-terminal kinase (JNK) signaling as a therapeutic target for Alzheimer's disease," *Frontiers in Pharmacology*, vol. 6, p. 321, 2015.

[60] P. Meyer, P. Maity, A. Burkovski et al., "A model of the onset of the senescence associated secretory phenotype after DNA damage induced senescence," *PLoS Computational Biology*, vol. 13, no. 12, Article ID e1005741, 2017.

[61] R. Gualtierotti, L. Guarnaccia, M. Beretta et al., "Modulation of neuroinflammation in the central nervous system: role of chemokines and sphingolipids," *Advances in Therapy*, vol. 34, no. 2, pp. 396–420, 2017.

[62] X. Ren, L. Zou, J. Lu, and A. Holmgren, "Selenocysteine in mammalian thioredoxin reductase and application of ebselen as a therapeutic," *Free Radical Biology and Medicine*, vol. 127, pp. 238–247, 2018.

[63] M. Mijit, V. Caracciolo, A. Melillo, F. Amicarelli, and A. Giordano, "Role of p53 in the regulation of cellular senescence," *Biomolecules*, vol. 10, no. 3, p. 420, 2020.

[64] N. Herranz, S. Gallage, M. Mellone et al., "mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype," *Nature Cell Biology*, vol. 17, no. 9, pp. 1205–1217, 2015.