Senegenin Rescues PC12 Cells with Oxidative Damage Through Inhibition of Ferroptosis

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Abstract
Oxidative stress is one of the pathological mechanisms of Alzheimer’s disease (AD), and ferroptosis has been determined to be involved in neurodegenerative diseases such as AD. Senegenin (Sen) prevents oxidative damage in nerve cells via a mechanism that may be highly related to ferroptosis. However, the mechanism of ferroptosis pathway involvement in AD is unclear. In this study, we established a model of PC12 cytotoxic injury induced by Aβ25–35, and we detected the level of oxidative damage, MMP, and ferroptosis-related protein expression. The results showed that, compared with control group, the level of ROS increased, GPX activities decreased, and MDA levels increased in Aβ25–35 group. Aβ 25–35 could induce mitochondrial depolarization in PC12 cells and Fer-1 could not reverse this damage. WB revealed that Aβ 25–35 group had increased ACSL4 and PEBP1 proteins, and decreased GPX4 protein. After adding Sen in the model, the level of oxidative damage was reduced, and mitochondrial depolarization was reversed compared with Aβ25–35 group. WB suggested that the expression of ACSL4 and PEBP1 proteins decreased, and the expression of GPX4 protein increased by Sen treatment. In conclusion, we found that Sen exhibits strong neuroprotective activity against Aβ25–35 induced oxidative damage and lipid metabolic associated with ferroptosis. Inhibiting nerve cell ferroptosis might facilitate the future development of strategies to AD.

Keywords Alzheimer’s disease · Aβ25–35 · Ferroptosis · Oxidative stress · Senegenin

Introduction
Alzheimer’s disease (AD) is the most common cause of senile dementia. It is reported that more than 10 million people are currently living with AD, and the incidence is growing rapidly with aging population [1]. Although studies have revealed potential mechanisms involved in the progression of AD, there is no good curative treatment strategy for dementia, such that patients still present with dementia symptoms [2]. Ferroptosis is a new form of programmed cell death characterized by excessive iron-dependent lipid peroxidation, which also plays an important role in the regulation of oxidative stress and inflammatory responses [3]. In fact, inflammation (not apoptosis) is a critical pathological feature of AD, and mitochondrial oxidative damage has been reported as early events in AD progression [4]. Therefore, the occurrence of ferroptosis in AD deserves more attention in future research.

In AD, abnormal metabolism of amyloid beta (Aβ) leads to accumulation of extracellular plaques formed by misfolded...
Aβ. In addition, Aβ deposits multiple organelles, like endoplasmic reticulum, Golgi apparatus, lysosomes, mitochondria, and cytosol [5]. Therefore, the toxicity of Aβ is involved in many specific physiological processes, including synaptic disruption, mitochondrial dysfunction, ubiquitin–proteasome system, and activation of pro-inflammatory responses [6].

Ferroptosis is characterized by lipid peroxides and reactive oxygen species [7]. It has reported that ferroptosis is related to the nerve cells protective in tumor cell killing and inhibition of excessive oxidative stress [8]. The increase of iron ion is associated with pathological cell death (Alzheimer’s, Huntington’s, and Parkinson’s diseases), canceration, stroke, cerebral hemorrhage, traumatic brain injury, ischemia–reperfusion injury, and renal degeneration [9]. Furthermore, iron imbalance can increase the production of reactive oxygen species (ROS), which is also one of the pathological features of AD [10], and lipid peroxidation of polyunsaturated fatty acids is the main type of oxidative damage in AD brain [11].

Senegenin (Sen), a Chinese herbal compound, is the major and most active ingredient of Radix Polygala. Sen shows unique pharmacological activity in promoting intelligence, anti-aging, antioxidant, and other aspects, and also possesses cytoprotective properties in neurodegenerative disorders [12, 13]. It has been previously reported that Sen was effective in inhibiting apoptosis and oxidative stress in Aβ1–42–induced PC12 cells [14]. Our previous study established that Sen could exert neuroprotection against H/R-induced injury via suppression apoptosis [15]. Furthermore, we found that RhoGDIα, which is a pharmacological target for Sen [16], is involved in the phosphorylation of tau and apoptosis in AD and VaD [17]. Here, we addressed the mechanism of Aβ25–35 in PC12 cells and found that Sen could rescue PC12 cells with oxidative damage through inhibition of ferroptosis.

**Material and Methods**

**Cell Culture**

Highly differentiated PC12 cells (rat pheochromocytoma cell line; Cell Bank of Chinese Academy of Sciences, Shanghai, China) in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a humidified incubator with 5% CO2 nourishment. The cell culture medium is changed for 2 to 3 days. PC12 cells were passaged with 0.125% trypsin–EDTA (newProbe) after trypsinization.

**Identification of PC12 Cells by Immunofluorescence Staining**

Fluorescence microscopy was used to identify highly differentiated PC12 cells. PC12 cells (2.5 × 10^5) were seeded in 6-well plates in each well, gently washed twice with ice-cold PBS, fixed in prewarmed 4% paraformaldehyde for 20 min at room temperature, washed with PBS, maintained in the wells for 10 min with 0.2% Triton X-100 solution, treated with 10% goat serum albumin for 30 min, incubated with a MAP-2 primary antibody (1:200) overnight in a humidified chamber at 4 °C, and treated with DyLight 488 AffiniPure goat anti-rabbit IgG for 1.5 h. The nuclei were stained with DAPI (1:200). Finally, the plates were washed in PBS, mounted with anti-fade mounting medium, and examined with an advanced IX71 inverted fluorescence microscope (Olympus Corporation, Japan).

**Cell Viability**

The lyophilized Aβ25–35 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Aβ25–35 of 1 mg was dissolved in 943 μL ultrapure water to form 1-mM-Aβ25–35 solution, which could be stored at −20 °C. Before Aβ25–35 was used for experimental applications, the 1-mM-Aβ25–35 solution was incubated with culture medium, maintaining at 37 °C for 2 weeks to obtain its aggregated form. The experimental concentration of Aβ25–35 was selected based on the CCK-8 assay outcome. PC12 cells were treated with increasing concentrations of Aβ25–35 (5 μM, 10 μM, 20 μM, 40 μM, 80 μM, and 160 μM) for 24 h, 36 h, and 48 h, and then the medium was replaced with 100 μL of fresh culture medium containing 10 μL of CCK-8 solution. Then, the plate was incubated for 2–4 h. The optical density (OD) values of the wells were measured by using a microplate reader at 450 nm. To determine the protective effect of Fer-1 (New Probe, China) against Aβ25–35–induced cell death, cell viability was detected using CCK assay. Increasing concentrations of Fer-1 (1.25 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, and 1600 μM) were added to PC12 cells to determine the safe dose of Fer-1, and experimental concentration of Fer-1 (1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM) were added to PC12 cells with Aβ25–35 treatment. The protective effect of Sen (New Probe, China) against Aβ25–35–induced PC12 cell death was determined. Increasing concentrations of Sen (15 μM, 30 μM, 60 μM, 120 μM, 240 μM, 480 μM, 960 μM, and 1920 μM) were added to PC12 cells to determine the safe concentration of Sen, and experimental concentrations of Sen (15 μM, 30 μM, 60 μM, 120 μM, and 240 μM) were added to PC12 cells with Aβ25–35 treatment. The concentrations of conditioned medium were selected by the same previously described method.

**Measurement of ROS Production**

The reagent dichloro-dihydro-fluorescein diacetate (DCF-HDA) (Nanjing Jiancheng Bioengineering, China) was used...
to determine intracellular ROS levels. After exposure to conditioned medium and Aβ25–35 for 24 h, the treated P12 cells were harvested and incubated with DCFH-DA (10 μM) for 30 min in the dark at 37 °C. Then, the PC12 cells were washed three times with D/F12. The fluorescence signals were recorded using a flow cytometer (ACEA Biosciences, Inc., USA), and representative images were obtained with a fluorescence microscope.

**Measurement of Intracellular Glutathione Peroxidase**

A glutathione peroxidase detection kit (Nanjing Jiancheng Bioengineering, China) was used to detect GSH-PX (glutathione peroxidase, GPX) activity. PC12 cells were seeded in 6-well plates at a density of 2.5 × 10^5 cells/well. After treatment with Aβ25–35 for 24 h, the PC12 cells were washed three times with PBS, 500 μL/well lysate to lyze cells for 30 min, and the culture supernatants were collected for measurement according to the manufacturer’s instructions. GPX activity = ([non-enzyme tube-enzyme tube absorbance] / [standard tube-blank tube]) × standard tube concentration (20 μmol/L) × dilution multiple (5 ×) / reaction time / (sample amount × sample protein content).

**Measurement of Intracellular Malondialdehyde**

Intracellular malondialdehyde test kit (Nanjing Jiancheng Bioengineering, China) was used to detect intracellular MDA (malondialdehyde) in PC12 cells. PC12 cells were seeded in 6-well plates at a density of 2.5 × 10^5 cells/well. After treatment with Aβ25–35 for 24 h, the PC12 cells were washed three times with PBS, and treated cells according to kit instructions. Then, the reaction solution was collected to 96-well plates, and the optical density (OD) values of the wells were measured by using a microplate reader at 530 nm.

**Mitochondrial Membrane Potential Measurement**

JC-1 existed either as a cytoplasmic JC-1 monomer or in mitochondrial JC-1 aggregates, depending on the MMP (mitochondrial membrane potential). The changes in MMP were measured with an MMP assay kit with JC-1 (Beyotime Biotechnology Co., China). PC12 cells were seeded in 6-well plates at a density of 2.5 × 10^5 cells/well. After treatment with Aβ25–35 for 24 h, the treated cells were harvested and washed with cold PBS one time. Then, the cells were suspended in a mixture of 1 mL of culture medium and 1 mL of JC-1 staining solution for 20 min in the dark at 37 °C. After incubation, the cells were absorbed and washed twice with cold JC-1 buffer; 2 ml of cell culture medium was added and visualized by fluorescence microscopy (Leica, Germany). The MMP was indicated by the ratio of red-to-green fluorescence intensity.

**WB Analysis**

PC12 cells from each group were washed with ice-cold PBS, and total protein was extracted with RIPA lysis buffer (Beyotime Biotechnology Co., China) containing 1% proteinase inhibitors (Bimake, USA). The protein concentrations were measured using a BCA protein assay kit (Beyotime Biotechnology Co., China). Equal amounts of protein samples were subjected to separation by 10% or 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Next, 5% non-fat slim milk was used to block the membranes. After washing three times with TBST, the primary antibodies against ACSL4, GPX4, PEBP1, and GAPDH (1: 1 000; Affinity) were used to perform WB analysis. The membranes were then incubated with the corresponding primary antibodies overnight at 4 °C. After the membranes were washed three times with TBST, they were incubated for 1 h at room temperature with goat anti-rabbit IgG polyclonal secondary antibody (1:5000–1:10,000). Lastly, the signals were measured with an enhanced chemiluminescence (ECL) kit (Millipore, USA) on a gel imaging system.

**Statistical Analysis**

All experiments were performed independently at least in triplicate. One-way analysis of variance (ANOVA) was used to analyze significant differences among groups. P values of less than 0.05 were considered to indicate statistical significance. All values are represented as the mean ± SEM, and all graphs were generated with GraphPad Prism software, version 5.0.

**Results**

**Identification of PC12 Cells and the Effect of Aβ25–35 on the PC12 Cells**

The proliferation ability of PC12 cells was observed under electron magnification microscopic. Normally growing differentiated PC12 cells were round, spindle, or irregular in shape, adhere to the wall, and have a clear boundary. The neurotoid features of PC12 cells, like strong refractive index and protuberant network, could be observed more clearly under high-power magnification (Fig. 1a). In addition, MAP2, a marker of mature neurons, can be clearly stained in well-differentiated PC12 cells, with a structure similar to neurons and axon dendrites under immunofluorescence staining (Fig. 1b).

Aβ25–35 cytotoxicity decreased cell viability, which was evaluated by CCK-8 assay. Compared with the control group, the cell viability of PC12 cells decreased as the
concentration of Aβ25–35 increases. When PC12 cells was treated with 20 μM Aβ25–35, the percentage of damaged cells was significantly higher, compared with the control group (Fig. 1c–e). Therefore, the 20 μM Aβ25–35-induced-PC12 cells were used in the following experiment.

**Oxidative Damage Induced by Aβ25–35 in PC12 Cells**

Since one of the characteristics of ferroptosis is the accumulation of lipid hydroperoxides, measuring lipid peroxidation is important for evaluating whether ferroptosis occurs [3]. Ferrostatin-1 (Fer-1) is an inhibitor of ferroptosis, which indicates the occurrence of ferroptosis [7]. The drug sensitivity and working concentration of different concentrations of Fer-1 to PC12 cells were determined by CCK8 method. The results suggested that 5 μM of Fer-1 could save the damage of PC12 cells caused by Aβ25–35 (Fig. S1).

The degree of oxidative damage were detected in PC12 cells before and after injury induced by 20 μM of Aβ25–35. The results showed that, compared with the control group, the level of intracellular reactive oxygen species was significantly higher in the Aβ25–35 group, and such phenomenon was reversed after Fer-1 treatment (Fig. 2a, b). GPX is a selenium-containing enzyme, which has an important anti-oxidant and antimicrobial function [18]. MDA concentrations were measured representing the level of lipid peroxidation [19]. The results showed that, compared with the control group, the Aβ25–35 group had lower GPX and higher MDA expression level. In contrast, compared with the model group, the expression levels of GPX and MDA were reversed by Fer-1 (Fig. 2c, d). These data demonstrated that Aβ25–35 could induce oxidative damage in PC12 cells and that Fer-1 could reverse this change to protect impaired cells.

**The Changes of Mitochondrial Membrane Potential and Lipid Metabolism-related Protein Levels**

The change of intracellular MMP could reflect the cellular features that are reminiscent of different stages of cell death to some extents [20]. Here, compared with the control group, decreased red fluorescence and increased green fluorescence in the Aβ25–35 groups (Fig. 3a, b) were observed by inverted fluorescence microscopy. Likewise, this phenomenon, which again was also observed in Aβ25–35 + Fer-1 group (Fig. 3a, b). These data demonstrated that Aβ25–35 could induce mitochondrial depolarization in PC12 cells, and Fer-1 could not reverse this damage.

Lipid metabolism disorder is an important feature of ferroptosis, so we determined whether the changes in the expression of lipid metabolism-related proteins by Western blotting (WB) (Fig. 3c). GPX4 is a key antioxidant enzyme that detoxifies lipid peroxides during ferroptosis. Compared with the control group, the expression of ACSL4 and PEBP1 proteins, which exert ferroptosis-promotive effect, were elevated (Fig. 3e, f), whereas the expression of GPX4 protein decreased (Fig. 3d) in the Aβ25–35 group. In contrast, the expression of ACSL4 and PEBP1 proteins decreased, and the expression of GPX4 protein increased significantly by Fer-1 treatment, further confirming Aβ25–35 induced ferroptosis in PC12 cells under oxidative stress.

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**Fig. 1 a:** Morphological observation of highly-differentiated PC12 cells under electron microscope; **b:** staining of neuronal marker MAP2 in highly-differentiated PC12 cells (Bar = 250 μm); **c, d, and e:** the cell viability of PC12 cells was subjected to different concentrations of Aβ25–35 by CKK-8 assay. (Mean ± SEM, n = 3). *P < 0.05 vs Control
Sen has the effects of neuronutrition and antioxidation. The viability of PC12 cells treated with different concentrations of Sen was measured by CCK8 method. The results suggested that the effects of different concentrations on PC12 cells were promoted at low concentration and inhibited at high concentration (Fig. S2a). When the concentration of Sen was 60 μM, it could resist the damage injury caused by Aβ25–35 to PC12 cells (Fig. S2b).

Compared with the model group, the accumulation of intracellular reactive oxygen species in the Sen group was significantly lower (Fig. 4a). Quantitative analysis of fluorescence properties of reactive oxygen was consistent with the observed before (Fig. 4b). Similarly, the level of GPX (Fig. 4c) and MDA (Fig. 4d) were reversed when Sen was added. From hereon, Sen showed good antioxidant capacity intimately associated with ferroptosis.

Sen Reversed Oxidative Damage Intimately Associated With Ferroptosis

To determine the effect of Sen on mitochondrial dysfunction, the MMP was detected by fluorescence microscopy and flow cytometry. The results showed that, compared with Aβ25–35 group, red fluorescence increased and green fluorescence decreased in the Aβ25–35 + Sen groups (Fig. 4e). Quantitative analysis of fluorescence properties of MMP was consistent with the observed before (Fig. 4f).

Sen-attenuated Ferroptosis by Altering Proteins Expression in Lipid Metabolic Pathways

On the basis of the strong protective effect provided by Sen and the possibility to lessen the oxidation injury of PC12 cells in the existence of Aβ25–35, we determined whether the expression of lipid metabolism-related proteins were attenuated by Sen treatment (Fig. 5a). As expected, compared with the model group, the expression of ACSL4 (Fig. 5b) and PEBP1 (Fig. 5d) proteins decreased, whereas the expression of GPX4 (Fig. 5c) protein increased in Aβ25–35 + Sen group.
This indicates that Sen not only attenuates the oxidative damage caused by Aβ25–35, but also affects the expression of ferroptosis-related proteins, which means that Sen has a certain inhibitory effect on the occurrence of ferroptosis.

Our data establish that Sen–ROS–ferroptosis pathway is a potent suppressor of Aβ25–35 and ferroptosis (Fig. 5e). Aβ25–35 induces intracellular oxidative stress and initiates ferroptosis. Ferroptosis inhibitor FER-1 can inhibit the occurrence and development of this process by upregulating the expression of GPX4. However, Sen can reduce ferroptosis of PC12 cells induced by Aβ25–35 and exert a cytoprotective effect. Its mechanism may be related to the upregulation of GPX4 protein expression, reduction of ROS, and MDA accumulation, and downregulation of ferroptosis-related proteins (ACSL4 and PEBP1) expression. As such, Sen, which have been shown to possess multiplex neuroprotective potential associated with AD, like Aβ aggregation [21], anti-inflammation [22], and antioxidant [23], may have different mechanisms worth exploring, like against ferroptosis. Furthermore, our discovery adds to the explanation for why lipid peroxidation is believed to be a convincing event in AD pathogenesis [10], and the production of ROS is also evident in AD brains [24]. Overall, Sen could attenuate oxidative damage caused by Aβ25–35, and ferroptosis is a major contributor of oxidative damage in PC12 cells.

**Discussion**

Synaptic loss and neuronal death are potential causes of neurodegenerative diseases, but the chronic brain pathological features associated with AD such as increased inflammation and degeneration, cannot be explained by apoptosis solely [2]. Studies have shown that there are other ways of cell death other than apoptosis in the brain of AD patients [25], which arouse more attention. Moreover, many studies have
documented oxidative stress in AD brain tissues, and lipid peroxidation of polyunsaturated fatty acids is the main type of oxidative damage in the AD brain [10]. Previous studies have reported that Sen can be used to treat AD. Sen has a strong effect on the scavenging and antioxidant capacity of free radicals in the body, and its mechanism is to reduce lipid peroxidation reaction [26]. However, the precise mechanisms of Sen which involved in lipid metabolic remain to be fully elucidated.

Ferroptosis proposed in 2012 lipid peroxidation and iron regulation disorders [7], a new cell death mechanism, which is genetically, morphologically, and biochemically different from apoptosis. Ferroptosis is involved in a variety of human diseases, like ischemic heart disease, renal failure, and other ischemic organ damage, and neurological diseases such as neurodegenerative diseases and brain damage [8]. Aβ has been pathologically identified and unequivocally accepted as the major hallmarks of AD, which can accumulate in synapses, affect and disrupt the signal pathway of actin dynamics in the pathogenesis of AD [27]. The previous study of our group found that the injury of PC12 cells induced by Aβ25–35 can simulate the damage of neurons in AD. On the basis of the above, we explored the changes of intracellular lipid metabolic which related to ferroptosis induced by Aβ25–35 in PC12 cells.

Our results demonstrated that Aβ25–35-induced ROS and other oxidation production multiply accumulated. Sen exhibits strong neuroprotective activity against oxidative damage and lipid metabolic associated with ferroptosis. When the levels of ROS exceed the antioxidant capacities of cells, oxidative stress occurs which often leads to cell death [28]. In addition to antioxidant activities, it has also been reported that Sen had a protective mechanism for mitochondria, mainly to protect the mitochondrial respiratory chain complex and ATP synthase from Aβ damage, and to further protect the integrity of the mitochondrial ultrastructure...
Studies have found that the occurrence of ferroptosis is accompanied by hyperpolarization of the MMP [29], which was not observed in this experiment, suggesting that the change of MMP during ferroptosis may be related to cell types and processing methods. Our previous study found that Aβ25–35-induced damage to PC12 will undergo apoptosis [15, 16], which is consistent with the results of this experiment. The typical metabolic activities of mitochondria, including the TCA cycle and the activity of the mitochondrial electron transport chain (ETC), are necessary to generate enough lipid ROS to initiate ferroptosis [29].

Furthermore, we detected the levels of lipid metabolism-related proteins (ACSL4, GPX4, and PEBP1). Acyl coenzyme long chain family (ACSL), a mainly expressed protein in endoplasmic reticulum and mitochondrial outer membrane, can catalyze fatty acids to form acyl coenzymes and act as intermediates in lipid metabolism to promote fatty acid metabolism and membrane modification [30]. The expression of ACSL4 (but not other ACSLs) is related to the sensitivity of cells to ferroptosis induced by erastin by producing 5-HETE-mediated adipotoxicity [31]. GPX4 is a glutathione peroxidase with a unique substrate preference, which can effectively reduce hydroperoxides in complex lipids, such as phospholipids, cholesterol and cholesterol esters [18]. It has been found that conditional removal of Gpx4 from adult mouse neurons may lead to rapid degeneration of spinal motoneurons through ferroptosis [32]. The authors further found that cognitive impairment and hippocampal neurodegeneration occurred in Gpx4, mice through forebrain-specific knockout [33], indicating that Gpx4 is closely related to ferroptosis. Phosphatidylethanolamine-binding protein 1 (PEBP1), known as Raf1 kinase inhibitory protein (RKIP1) is bound to and inhibits the Raf1 kinase cascade under homeostatic conditions. Studies have demonstrated PEBP1-dependent regulatory mechanisms of ferroptotic by binding with lipoxygenases and allowing them to generate lipid peroxides [34].

Fer-1, a ferroptosis inhibitor, inhibits the production of ROS and reduces the level of intracellular oxidative stress...
[7]. Under Aβ25–35 injury, Fer-1 significantly reversed oxidative damage and lipid metabolism-related protein expression, which was a strong evidence of ferroptosis. Therefore, Sen exhibits strong neuroprotective activity against Aβ25–35-induced oxidative damage and lipid metabolic through inhibition of ferroptosis.

In summary, our research results show that there is not only apoptosis but also ferroptosis in PC12 cells injured by Aβ25–35. Sen can play a positive role in AD by reducing the level of oxides and inhibiting the occurrence of ferroptosis. Although the global incidence of AD is still severe and treatment options are limited, we believe that revealing more pathogenesis of AD, such as the occurrence of ferroptosis, can provide new ideas for the containment and treatment of AD.

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Author Contribution All the authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Heping Zhang, Wei Zhou, Jianling Li, and Zhaohui Qiu. The first draft of the manuscript was written by Heping Zhang, and all the authors commented on the previous versions of the manuscript. All the authors read and approved the final manuscript.

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Data Availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Not applicable to this study.

Consent to Participate Not applicable to this study.

Consent for Publication Not applicable to this study.

Competing Interests The authors declare no competing interests.

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