ORIGINAL RESEARCH

Senegenin Inhibits Aβ$_{1-42}$-Induced PC12 Cells Apoptosis and Oxidative Stress via Activation of the PI3K/Akt Signaling Pathway

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Background/Aim: Apoptosis and oxidative stress have been considered as key events in the pathogenesis of Alzheimer's disease (AD). Senegenin (Sen), the major and most effective ingredient of *Radix Polygalae*, which has anti-apoptotic and anti-oxidative effects. The aim of this study was to investigate the anti-apoptotic and anti-oxidant effects of Sen on Aβ$_{1-42}$-induced PC12 cells apoptosis and oxidative stress as well as its possible signaling pathway.

Methods: Rat pheochromocytoma (PC12) cells were treated by 20 μM Aβ$_{1-42}$ and then divided into 5 different treatment groups (Control; Aβ$_{1-42}$ 20 μM; Aβ$_{1-42}$ 20 μM + Sen 10 μM; Aβ$_{1-42}$ 20 μM + Sen 30 μM; Aβ$_{1-42}$ 20μM + Sen 60 μM). PC12 cells activity was detected by MTT assay. Colony formation assay was performed to assess the clonogenic ability of cells. The cell apoptosis was detected by Annexin-V/PI staining. The pro-apoptotic protein (Bax), anti-apoptotic protein (Bcl-2), anti-oxidative stress factor (HO-1, Nuclear Nrf2, Total Nrf2) and pathway-related protein (Akt, P-Akt, PI3K, P-PI3K) were tested by Western blot. The reactive oxygen species (ROS) level was assessed with a DCFH-DA probe.

Results: The results indicated that Sen dose-dependently increased cell viability and reduced the number of apoptotic cells. The ratio of P-PI3K/PI3K and P-Akt/Akt increased in a dose-dependent manner under the treatment of Sen, suggesting that Sen might activate the PI3K/Akt signaling pathway. Moreover, Sen upregulates the ratio of Bcl-2/Bax. Further study revealed that Sen can play an antioxidant role in enhancing HO-1, promoting Nrf2 nuclear translocation and reducing ROS accumulation to reduce oxidative stress.

Conclusion: Sen is effective in inhibiting apoptosis and oxidative stress in Aβ$_{1-42}$-induced PC12 cells, which likely contribute to the development of novel therapies for AD.

Keywords: Alzheimer's disease, senegenin, apoptosis, Aβ, oxidative stress, PC12 cells

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, accounting for approximately 60% to 80% of cases.[1] Although the pathogenesis of AD is still unclear, β-amyloid (Aβ) deposition, neurofibrillary tangles and neuronal loss have been found to be closely related to cognitive dysfunction in patients.[2–4] Numerous studies have demonstrated the toxicity of Aβ in vitro and in vivo, causing neuronal loss, tau phosphorylation and microglial proliferation[5–9] and can lead to neuronal cell death.[10] Two major forms of Aβ, such Aβ$_{1-42}$ and Aβ$_{1-40}$, considered as a disease marker in AD. As Aβ$_{1-42}$ is more prone to aggregation than Aβ$_{1–40}$ and the accumulation and precipitation of Aβ$_{1-42}$ was considered a neuropathological event s associated with the progression and development of AD.[11,12] Notably, the mechanism of Aβ induced cell death is still controversial. Some studies believe that Aβ inhibit cell viability and increase the percentage of DNA damage through necrosis.[13–15] However, other studies suggest that apoptosis is the main mechanism of Aβ-induced
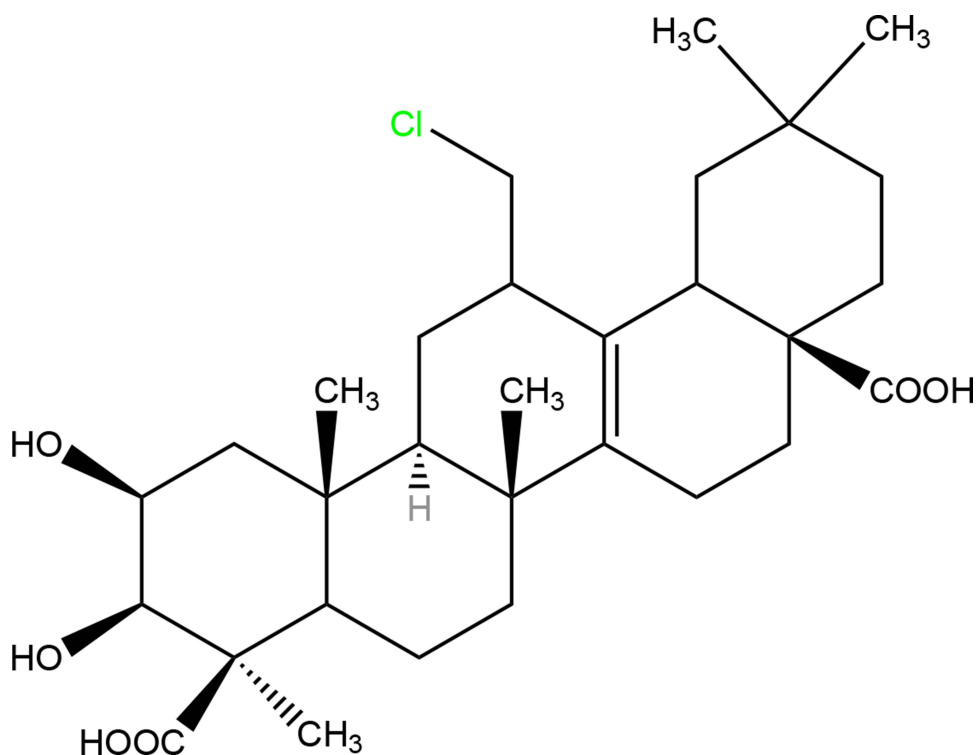
Figure 1 Chemical structure of senegenin.

neuronal death.[16–18] In our study, we are more support the mechanism of apoptosis, but the pathway and mechanism of Aβ-induced apoptosis still need to be further studied.

The phosphatidylinositol 3-kinases (PI3Ks), a family of intracellular lipid kinases, are crucial roles in the upstream of the PI3K/Akt signaling pathway.[19] The PI3K/Akt signaling pathway is directly regulated by Aβ exposure, and many downstream factors have an important impact on AD.[20–22] Furthermore, the PI3K/Akt signaling pathway, as a key regulator of apoptosis, is believed to play a vital role in AD.[23–25] In the apoptotic pathway, Bax and Bcl-2 are two critical downstream target proteins.[26] PI3K/Akt signaling pathway promotes or inhibits Bcl-2 and Bax by phosphorylation.[27] Therefore, it was important to study the expression of Bcl-2 and Bax to explore apoptosis. In addition, there is substantial evidence supporting that oxidative stress is a common pathogenesis of AD.[28,29] Oxidative stress can induce excessive production of reactive oxygen species (ROS) and excessive ROS also promote oxidative stress, ultimately, cell death.[30,31] ROS is induced by PI3K/Akt pathway, which triggers various intracellular reactions.[32,33] Moreover, PI3K/AKT pathway may be an important way to activate/synthesize various antioxidant factors, such as Nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1).[34–36] Therefore, drugs that block apoptosis and oxidative stress may be useful in preventing neuronal cell death and treating AD.[17,37–39]

Senegenin (Sen) is the active component of *Polygala tenuifolia* root, a traditional herbal medicine that has been widely used in China (Figure 1). Biochemical analysis has proved that the active component of *P. tenuifolia* are mainly saponins derived from presenegenin. Current research shows that *P. tenuifolia* has neuroprotective and intellectual promoting activities, which can safely and effectively reduce memory damage[40,41] and reduce Aβ secretion.[42,43] Moreover, a previous study has showed that Sen has anti-apoptosis and anti-oxidation effects in hypoxia or reoxygenation injury.[44]

In the present study, we focused on the effects of Sen on apoptosis and oxidative stress in PC12 cells, as well as its possible pathway and the underlying mechanism. This may provide new evidence for a better understanding of the neuroprotective mechanism of Sen.

Materials and Methods

Materials

$A\beta_{1\text{-}42}$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Senegenin (purity>98%) was purchased from Mansite Pharmaceutical Co., LTD. (Chengdu, China). Dulbecco's modified Eagle medium (DMEM), penicillin, fetal bovine serum (FBS) and streptomycin were purchased from Solarbio (Beijing, China). The Annexin-V/PI staining kit was purchased from KeyGEN (Nanjing, China). 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) probe and Nuclear and Cytoplasmic Protein Extraction Kit were purchased from Beyotime Biotechnology (Shanghai, China). Antibodies against Akt (cat.no.ab18785), P-Akt (cat.no. ab38449), PI3K (cat.no. ab139307), P-PI3K (cat.no. ab182651), Bax (cat.no. ab232479), Bcl-2 (cat.no. ab59348), Nrf2 (cat.no. ab62352) and β-actin (cat.no. ab179467) were from Abcam (Cambridge, United Kingdom). HO-1 (cat.no. GTX101147) was from Gene Tex (Irvine, CA, USA) and Lamin A (cat.no. 201015-3D6) was from Zen-Bio (Chengdu, China).

Cell Culture and Drug Treatment

Rat pheochromocytoma (PC12) cells were obtained from the Chinese Academy of Sciences. The cells were maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% CO^2 incubator, the medium was changed every other day. $A\beta_{1-42}$ was dissolved in PBS and incubated at 37°C for seven days to induce aggregation.[45,46] Next, the aggregated $A\beta_{1-42}$was diluted to the desired concentration. A 2mM stock solution of Sen was prepared by dissolving 20mg Sen powder in 15mL DMEM and placed at −20°C for long-term storage. Working solutions were diluted from the stock solutions at an appropriate concentration for further use.

Cell Viability Assay

PC12 cells were seeded into 96-well plates (0.5×10^4cells/well) and assessed for response to $A\beta_{1-42}$ and Sen using the MTT assay. Briefly, the cells were pretreated with Sen (0, 10, 30, 60 or 80 μM) only for 1 h and then exposed to $A\beta_{1-42}$ for 24 h in the continued presence of Sen (0, 10, 30, 60 or 80 μM). After incubations, PC12 cells were processed with 5 mg/mL MTT for 4 h at 37°C and the medium was carefully removed. Formazan crystals were dissolved in 150 μL DMSO. The absorbance at 570 nm was read using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA) which correlated to the viability of cells.

Colony Formation Assay

To assess the clonogenic ability of cells, a colony formation assay was performed. The cultured PC12 cells were plated before the treatment with $A\beta_{1-42}$. Cells were harvested from a stock culture and plated at proper dilutions into dishes. After the attachment of the cells to the dishes, which generally takes 2 h or more, the cells were treated with 20μM of $A\beta_{1-42}$ and co-treated with different concentrations of Sen (0, 10, 30 or 60 μM). After treatment, place the dishes in the incubator for at least 2–3 weeks. The colonies were stained with Giemsa stain solution. Clones containing more than fifty cells were counted under an inverted microscope (Nikon, Tokyo, Japan).

Apoptosis Assays

Apoptosis was tested using the Annexin V-FITC/PI double staining Kit (Keygen Biotech Co., Nanjing, China). In the end of incubation, PC12 cells were washed in cold PBS then centrifuged twice at 2000 rpm for five minutes and resuspended in 500 μL of binding buffer. Then FITC labeled Annexin V (5μL) and propidium iodide (PI, 5μL) were added to the cells, and then incubated for 20 minutes at room temperature in the dark according to the manufacturer's instructions. The cells were analyzed by flow cytometry (Becton Dickinson, USA).

Western Blot Analysis

Firstly, PC12 cells were dissolved with RIPA buffer. Nuclear and cytoplasmic proteins were extracted using Nuclear and Cytoplasmic Protein Extraction Kit. Protein concentrations were measured by the bicinchoninic acid protein assay kit

according to the protocol provided by the manufacturer (CoWin Biosciences Co., Ltd., China), and 100μg of total protein was separated by 10% SDS-PAGE electrophoresis. Then the proteins were transferred to a PVDF membrane. The membrane was blocked with 5% milk for 1 h at room temperature, and the primary antibodies (P-Akt, Akt, PI3K, P-PI3K, Bax, Bcl-2, HO-1, Nuclear Nrf2, Total Nrf2, LaminA and β-actin) were incubated at 4°C overnight. Chemiluminescence was used for color development, and image analysis was calculation with Quantity One software version 4.4.6 (Media Cybernetics, Inc., Rockville, MD, USA).

Determination of Intracellular ROS

To measure intracellular ROS level in PC12 cells after exposure to the oxidation-sensitive fluorescent dye DCFH-DA. The fluorescence was detected by the microplate reader, the excitation of which was set as 488 nm and the emission as 525 nm. 1×10^4 PC12 cells per well were seeded in 96-well culture plates. Cells were pretreated with Sen (0, 10, 30 or 60μM) for 1 h then exposed to Aβ$_{1-42}$ (20μM) for 24 h. Subsequently, DCFH-DA (10 μM) treated cells for 30 min at 37°C in the dark and cells were washed with PBS. The fluorescence was detected by the fluorescence microplate reader.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and analyzed by SPSS 26.0 statistical software (SPSS Inc., Chicago, IL, USA). Each procedure was performed in duplicate in three independent experiments. Statistical analyses were performed using analysis of variance (one-way ANOVA) and Student's t test was used when two groups were compared. Data were considered significant at P <0.05.
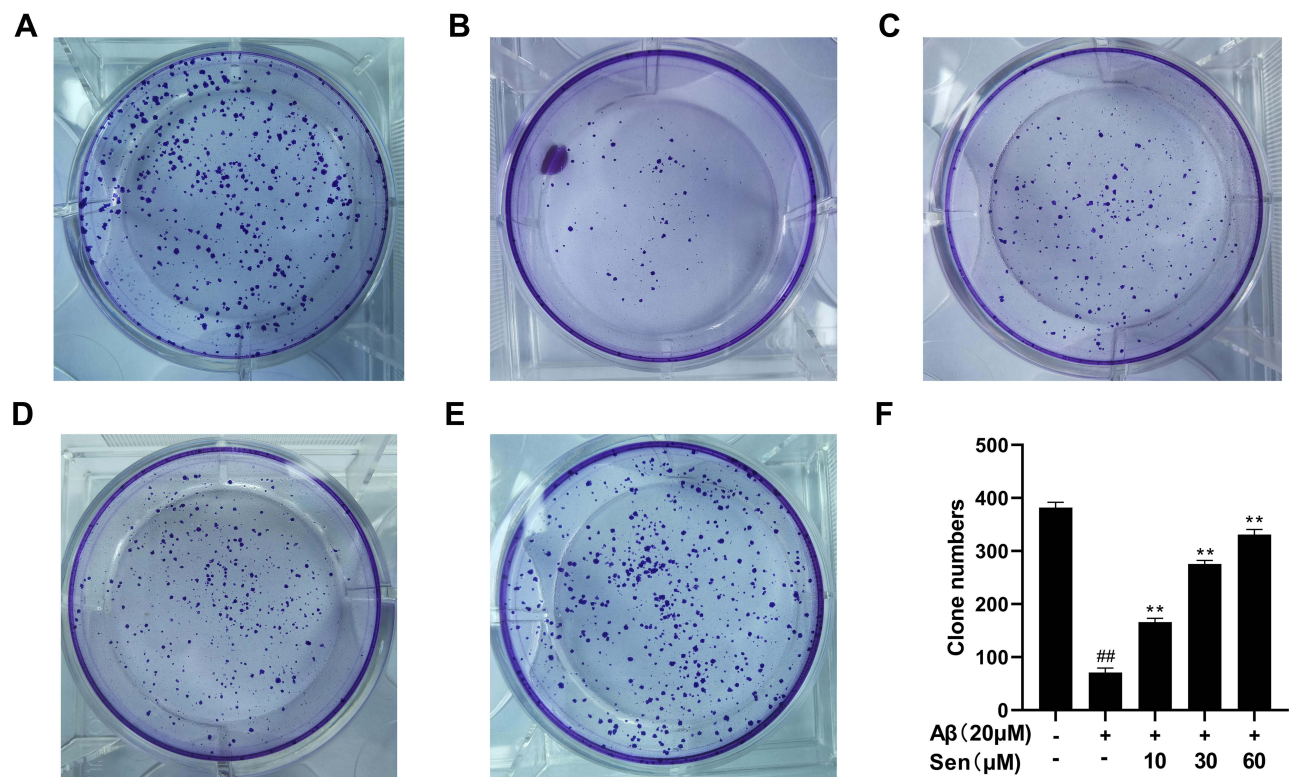
Results

Sen Reduced Aβ$_{1-42}$-Induced Toxicity of PC12 Cells

To assess the impact of Sen on Aβ-induced apoptosis, we first to assess the dose response to Aβ$_{1-42}$ of PC12 cell viability by MTT assay. Cells were treated with 0, 10, 20, 30, or 40 μM Aβ$_{1-42}$ as shown in Figure 2A, 20 μM Aβ$_{1-42}$ significantly decreased PC12 cell viability and 40 μM Aβ$_{1-42}$ resulted in the minimal cell viability of 53.5%, indicating a dose-dependent effect.

Next we assessed the effect of Sen alone on cell viability, which showed no statistical difference at Sen concentrations ranging from 0 to 60 μM (Figure 2B). Previous studies have shown that cells with activity of about 70% will be selected for the next experiment, which with 20 μM Aβ.[25,47–49] For these studies, we explored the effect of Sen on cell viability in PC12 cells treated with 20 μM Aβ$_{1-42}$. To determine the optimal dose of Sen, we first incubated PC12 cells with 10, 30,
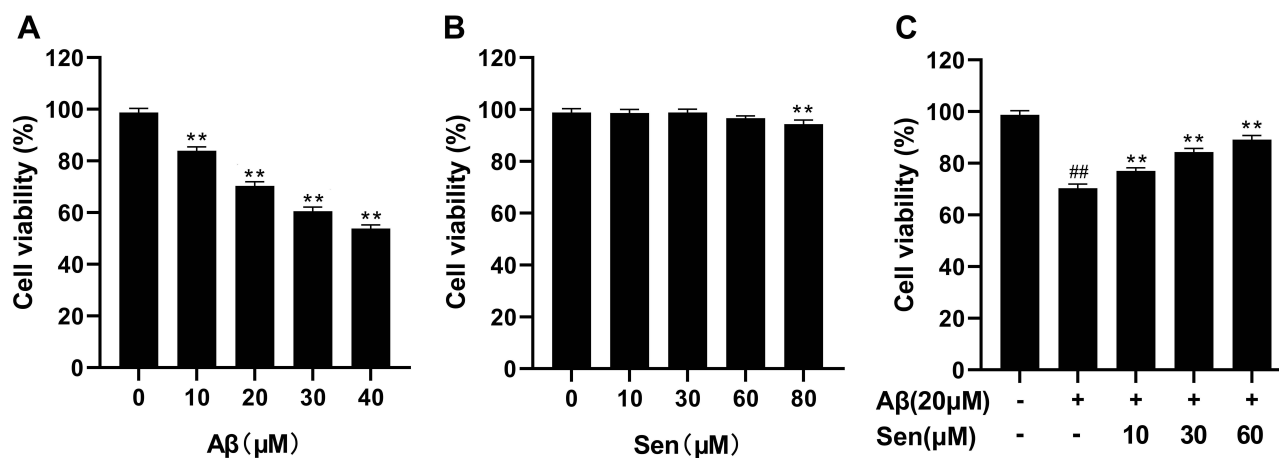
Figure 2 Sen protects Aβ$_{1-42}$-induced cytotoxicity in PC12 cells. (**A**) Effects of Aβ$_{1-42}$ on cell viability. PC12 cells were treated with 0–40 μM Aβ$_{1-42}$, and cell viability was assessed by the MTT assay. (**B**) Effects of Sen on cell viability. PC12 cells were treated with 0–80 μM Sen, and cell viability was assessed by the MTT assay. (**C**) Protective effect of Sen against Aβ$_{1-42}$-induced cytotoxicity in PC12 cells. After 1 h pretreatment with Sen (0, 10, 30, 60 or 80 μM), PC12 cells were treated with 20 μM Aβ$_{1-42}$ for 24 h. Results are presented as mean ± SD of three independent experiments. **P<0.01 versus Aβ$_{1-42}$ treatment group; ##P<0.01 versus control group.

Figure 3 The clonogenic ability was analyzed by colony formation assay. (**A**) Control; (**B**) Aβ$_{1-42}$ 20 μM; (**C**) Aβ$_{1-42}$ 20 μM + Sen 10 μM; (**D**) Aβ$_{1-42}$ 20 μM + Sen 30 μM; (**E**) Aβ$_{1-42}$ 20μM + Sen 60 μM; the results were integrated into (**F**). All data are presented as mean ± SD of three independent experiments. **P<0.01 versus Aβ$_{1-42}$ treatment group; ##P<0.01 versus control group.

60μM of Sen for 1 h, respectively, before adding 20 μM Aβ$_{1-42}$. We found that Sen induced an increase in PC12 cell viability in a dose-dependent manner (Figure 2C). Subsequently, the proliferation of PC12 cells was analyzed by colony formation assay. Aβ$_{1-42}$ has a significant inhibitory effect on cell proliferation and Sen significantly restored the proliferation of Aβ$_{1-42}$-treated PC12 cells. Colony formation assay further confirmed the neuroprotective effect of Sen (Figure 3).

## Sen Decreased Aβ$_{1-42}$-Induced Apoptosis Rate in PC12 Cells

Apoptosis rate was detected with Annexin V/PI double staining. Our data indicated that 20 μM Aβ$_{1-42}$ was sufficient to induce PC12 cell apoptosis with a rate of 64.27% by 24h. However, pretreatment with Sen (10, 30, 60 μM) for 1 h prior to Aβ$_{1-42}$ exposure decreased the apoptotic rate to 35.65%, 26.25% and 15.74% in a dose-dependent manner, respectively (Figure 4). The results imply that Sen is anti-apoptotic in PC12 cells.

Sen Increase the Protein Expression of Nrf2 and HO-1

In order to examine the effect of Sen on oxidative stress, we analyzed HO-1 and Nrf2 by Western blot analysis. Compared with control group, the protein levels of HO-1, Nuclear Nrf2, and Total Nrf2 were significantly decreased in Aβ$_{1-42}$ treatment group, while in Sen treatment groups, the protein expression of HO-1, Nuclear Nrf2, and Total Nrf2 was significantly increased in a dose-dependent manner (Figure 5). The result exhibited Sen is resistant to oxidation.

Sen Increased the Bcl-2/Bax Ratio

As shown in Figure 5, we detected the expression of anti-apoptosis protein Bcl-2 and pro-apoptosis protein Bax in different treatment groups. Bcl-2/Bax ratio in the Aβ$_{1-42}$ treatment group was decreased compared with the control group, but after Sen treatment, this ratio was increased compared with the Aβ$_{1-42}$ treatment group in a dose-dependent manner (Figure 6). The results indicated that Sen confers an anti-apoptotic effect under Aβ$_{1-42}$.
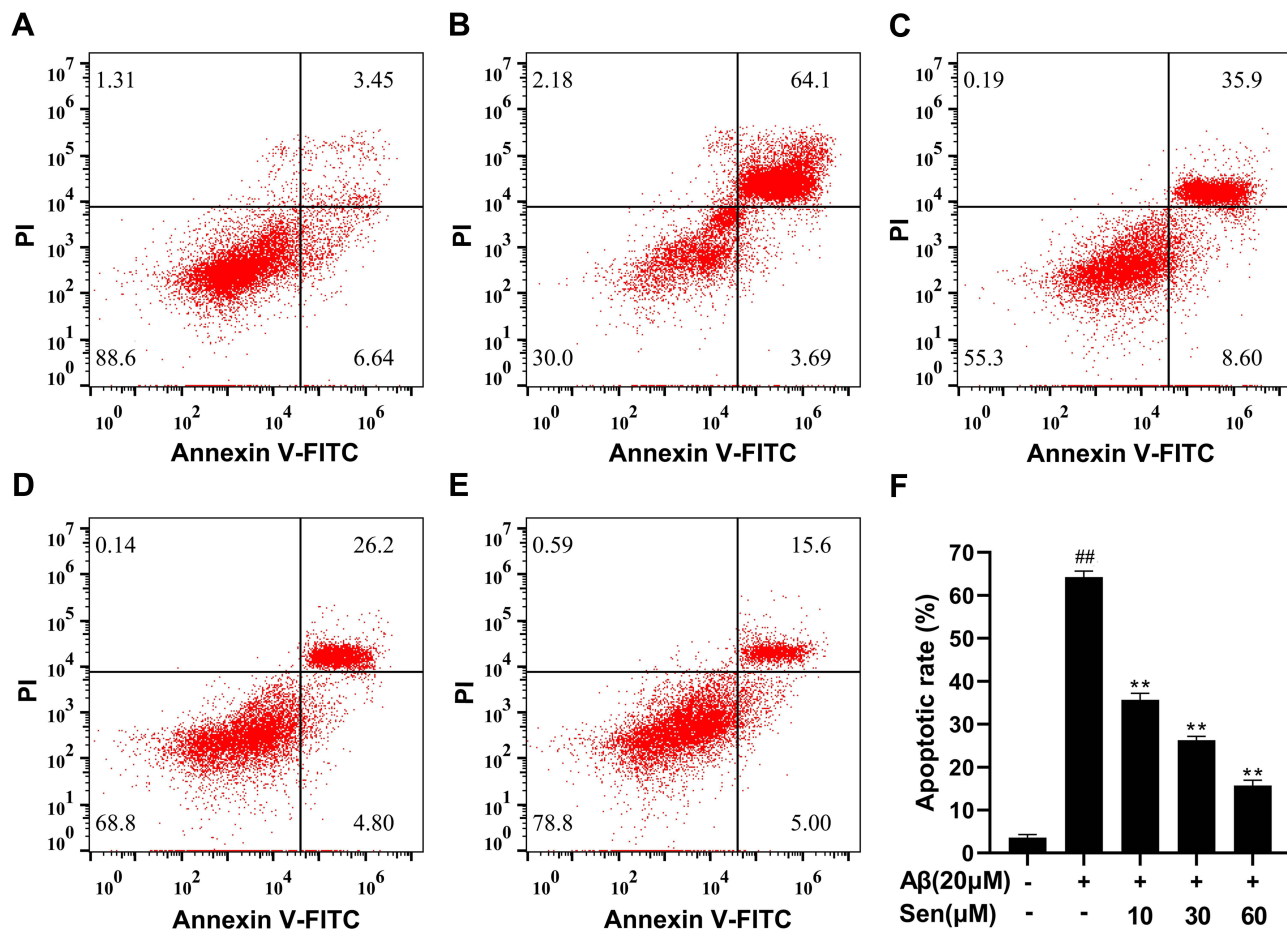
Figure 4 Protective effects of Sen on $A\beta_{1-42}$-induced apoptosis. Cell apoptosis were measured by Annexin-V/PI staining. (**A**) Control; (**B**) $A\beta_{1-42}$ 20 μM; (**C**) $A\beta_{1-42}$ 20 μM + Sen 10 μM; (**D**) $A\beta_{1-42}$ 20 μM + Sen 30 μM; (**E**) $A\beta_{1-42}$ 20μM + Sen 60 μM; the results were integrated into (**F**). All data are presented as mean ± SD of three independent experiments. **P<0.01 versus $A\beta_{1-42}$ treatment group; ##P<0.01 versus control group.

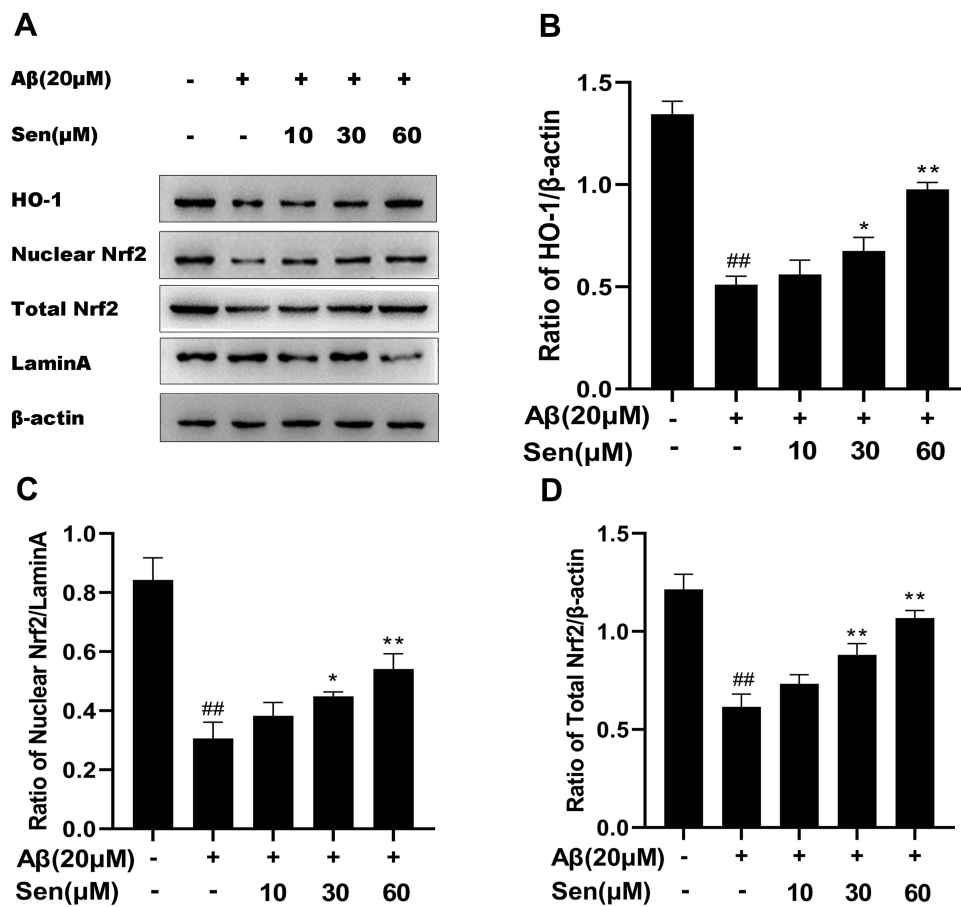## Sen Attenuated $A\beta_{1-42}$-Induced Accumulation of Intracellular ROS Production

We investigated the effect of Sen on $A\beta_{1-42}$-induced ROS by DCFH-DA staining. The results demonstrated that intracellular ROS levels significantly increased by $A\beta_{1-42}$ stimulation, and significantly decreased after treated with Sen in a dose-dependent manner (P<0.01). These results indicate that Sen may has anti-oxidative effect by attenuating intracellular ROS levels in PC12 cells (Figure 7).

## Sen Activated the PI3K/Akt Pathway

To examine the effects of Sen on PI3K phosphorylation (P-PI3K) and Akt phosphorylation (P-Akt), we analyzed P-PI3K and P-Akt by Western blot analysis. As shown in Figure 8, the ratio of P-PI3K/PI3K and P-Akt/Akt in the $A\beta_{1-42}$ treatment group was decreased compared with the control group. Sen increased in the ratio of P-PI3K/PI3K and P-Akt/Akt compared to the $A\beta_{1-42}$ group in a dose-dependent manner (p < 0.01). These results demonstrated that Sen may activate the PI3K/Akt signaling pathway, providing evidence that PI3K/Akt pathway may play an important role in the protective effects of Sen.

Discussion

Extensive neuronal cell death is an important hallmark of AD.[50–52] Although the pathological mechanism is not clear, preventing nerve cell death is a promising method for the treatment of AD.[53] The PC12 cell line has been widely used as an in vitro model to explore mechanisms in AD. In addition, the cells are particularly sensitive to Aβ-induced injury.[54,55]

A

| Aβ(20μM) | - | + | + | + | + |
| Sen(μM) | - | - | 10 | 30 | 60 |

HO-1

Nuclear Nrf2

Total Nrf2

LaminA

β-actin

B

C

D

Figure 5 Sen stimulates activation of Nrf2 and HO-1 in PC12 cells. HO-1, Nuclear Nrf2 and Total Nrf2 protein were detected by Western blotting. (**A**) Representative protein bands of HO-1, Nuclear Nrf2 and Total Nrf2; (**B**) the expression ratio of HO-1/β-actin; (**C**) the expression ratio of Nuclear Nrf2/LaminA; (**D**) the expression ratio of Total Nrf2/β-actin. Results are presented as mean ± SD of three independent experiments. *P<0.05, **P<0.01 versus Aβ$_{1-42}$ treatment group; ##P<0.01 versus control group.

A

| Aβ(20μM) | - | + | + | + | + |
| Sen(μM) | - | - | 10 | 30 | 60 |

Bcl-2

Bax

β-actin

B
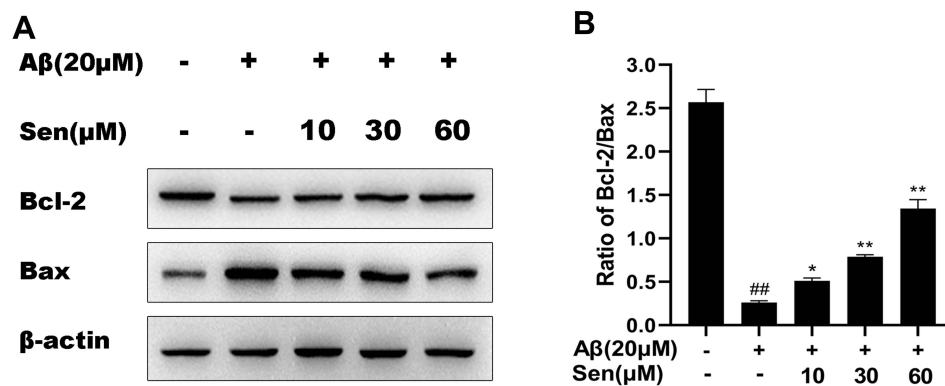
Figure 6 The level of Bcl-2/Bax was detected by Western blot. (**A**) Representative protein bands of Bcl-2 and Bax. (**B**) The expression ratio of Bcl-2/Bax. Data was expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01 versus Aβ$_{1-42}$ treatment group; ##P<0.01 versus control group.

In the present study, we established a PC12 cell model induced by Aβ$_{1-42}$ to explore the neuroprotective effects and potential mechanisms of Sen.

Apoptosis is a common physiological phenomenon that leads to cell death.[56,57] The PI3K/Akt signaling pathway, as a key regulator of apoptosis, is believed to play a vital role in AD.[23–25] Increasing the phosphorylation of PI3K and Akt can inhibit the apoptosis, on the contrary, promote the apoptosis.[58,59] The Bcl-2 family proteins are classified into anti-
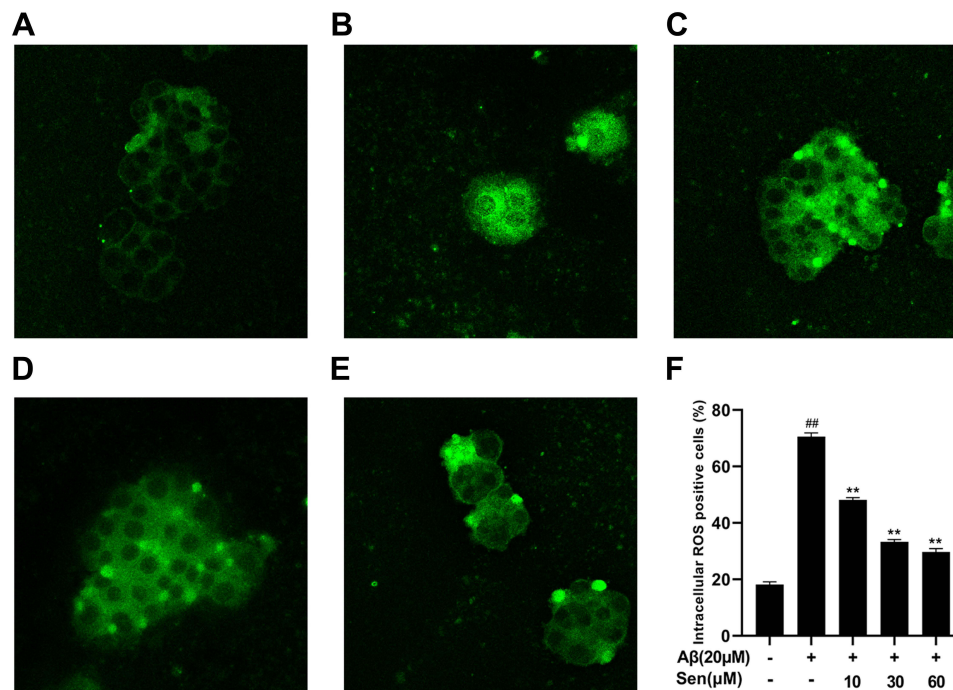
Figure 7 Sen prevented abnormal increases in intracellular ROS levels in $A\beta_{1-42}$-induced PC12 cells. Intracellular ROS levels were determined using DCFH-DA staining after treatment with Sen and $A\beta_{1-42}$ in PC12 cells. (**A**) Control; (**B**) $A\beta_{1-42}$ 20 μM; (**C**) $A\beta_{1-42}$ 20 μM + Sen 10 μM; (**D**) $A\beta_{1-42}$ 20 μM + Sen 30 μM; (**E**) $A\beta_{1-42}$ 20μM + Sen 60 μM; the results were integrated into (**F**). Results are presented as mean ± SD of three independent experiments. **P<0.01 versus $A\beta_{1-42}$ treatment group; ##P<0.01 versus control group.
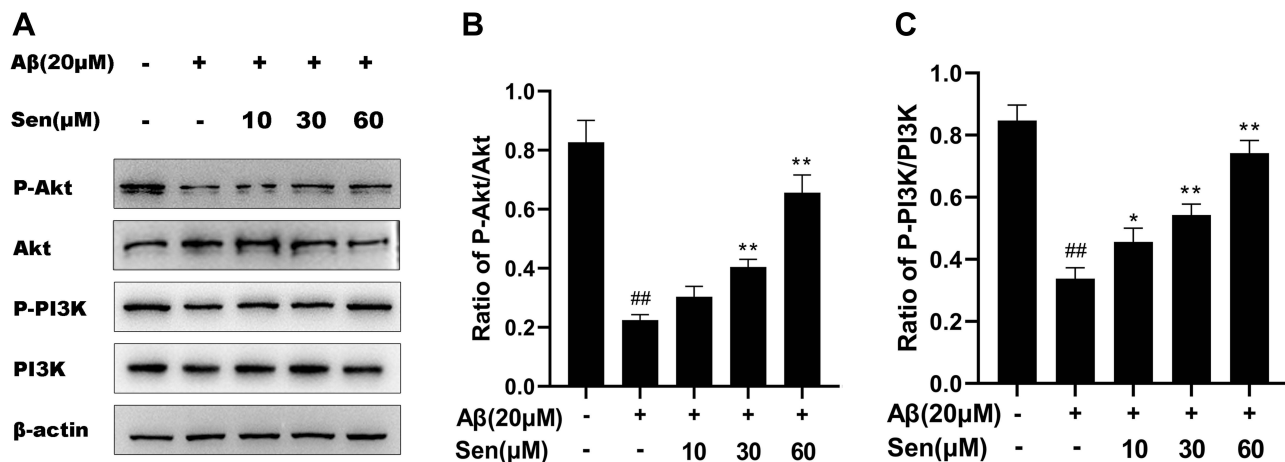

Figure 8 Expression levels of the PI3K/AKT pathway were examined in PC12 cells. The level of P-Akt/Akt and P-PI3K/PI3K were detected by Western blot and analyzed. (**A**) Representative protein bands of P-Akt, Akt, P-PI3K and PI3K; (**B**) the expression ratio of P-Akt/Akt; (**C**) the expression ratio of P-PI3K/PI3K. All data are presented as mean ± SD of three independent experiments. *P<0.05, **P<0.01 versus $A\beta_{1-42}$ treatment group; ##P<0.01 versus control group.

apoptotic proteins, including Bcl-2, and pro-apoptotic proteins, such as Bax.[60,61] Bcl-2 and Bax are two key downstream target proteins in the PI3K/Akt signaling pathway.[26] The ratio of Bcl-2/Bax determines whether or not a cell could undergo apoptosis.[62,63] In this study, we found that $A\beta_{1-42}$ treatment decreased the ratio of P-PI3K/PI3K, P-Akt/Akt and Bcl-2/Bax. Notably, Sen could increasing the phosphorylation of PI3K and Akt, which was consistent with the previous study.[64] Therefore, Sen may reduce apoptosis by regulating the expression of Bcl-2 family proteins.

Oxidative stress is designated as an imbalance between the production of ROS and antioxidant defenses, which may be induced by exposure to Aβ and thought to contribute to memory decline.[65,66] ROS may be induced by PI3K/Akt pathway[32,33] and mediates Aβ-induced neurotoxicity in the neurodegenerative process of AD.[67,68] Considering the
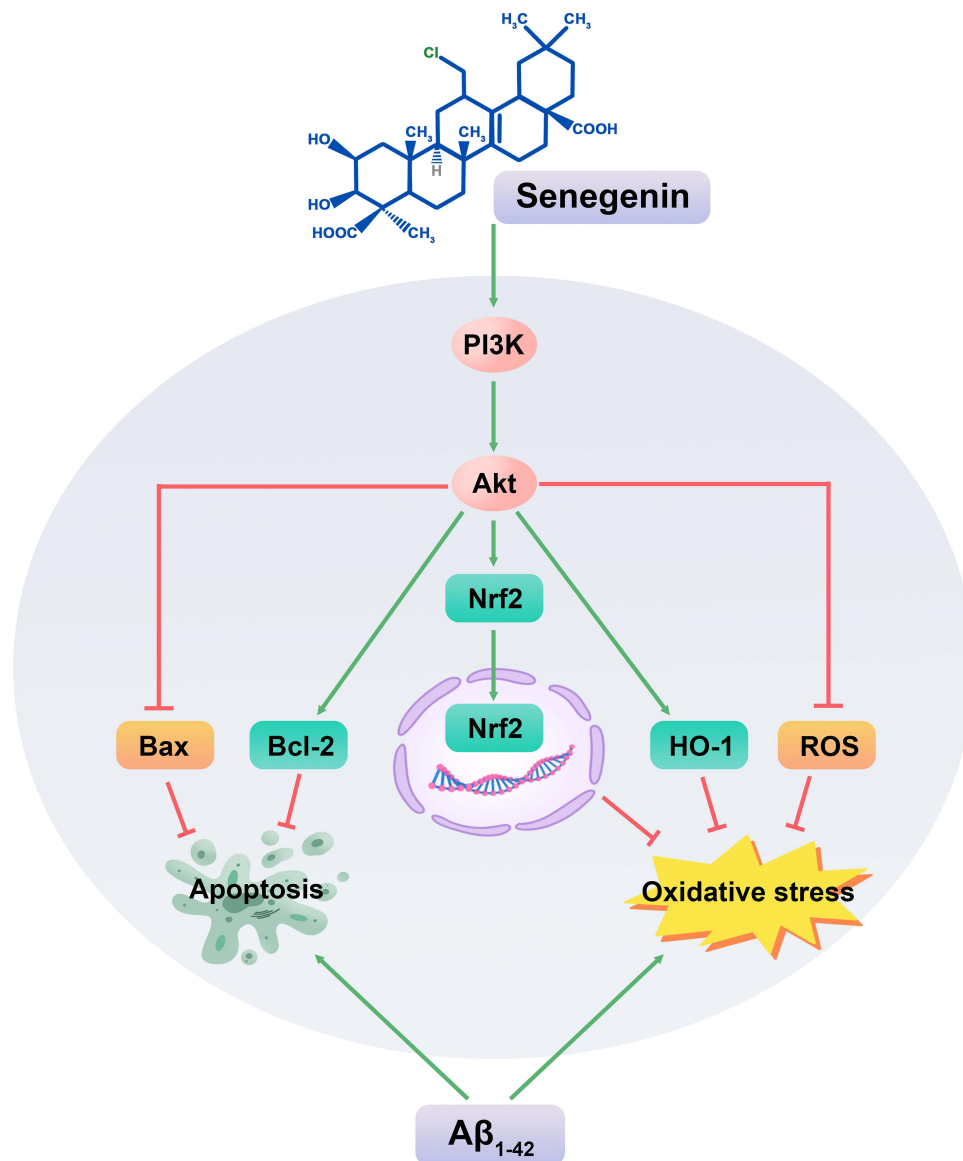
Figure 9 Schematic diagram of the possible mechanism underlying the neuroprotective effects of Sen on PC12 cells.

important role of ROS in Aβ-induced neurotoxicity, compounds with antioxidant effects may have a potential neuro-protective effect on AD. Consistent with a previous research study, our results also showed that PC12 cells treated with Sen have been found to inhibit the intracellular production of ROS.[44] In addition, Sen activated PI3K/Akt pathway, so we speculate that the antioxidant effect of Sen may related with PI3K/Akt pathway.

Nrf2 is a transcription factor and it has been shown to produce obvious antioxidant effects.[69] When stimulated by external oxidative stress, Nrf2 will transfer to the nucleus and activate various protective molecules, thereby enhancing the cell's resistance to external stimulation.[70] In addition, Nrf2 can regulate the coordinated expression of cytoprotective genes, including enzymes such as HO-1.[71] Pharmacological induction of HO-1 or overexpression of HO-1 in neuronal cells may have a protective effect on oxidative stress.[72,73] Previous studies demonstrated that the PI3K/Akt signaling pathway has been demonstrate to be part of a central pathway involved in the activation and translocation of Nrf2 for highly specialized protein synthesis, including HO-1.[34–36] In this study, the results showed that Sen that may be achieved by upregulating the levels of PI3K/Akt phosphorylation increased the expressions of HO-1 and promoted Nrf2 translocation into the nucleus in a dose-dependent manner.

Conclusion

In summary, this study provides evidence that Sen may be effective in inhibiting apoptosis and oxidative stress in $A\beta_{1-42}$-induced PC12 cells, which might occurs by activating the PI3K/Akt signaling pathway. We describe in Figure 9 the possible molecular mechanism of Sen in exerting its neuroprotective effects in PC12 cells. Further researches are needed to better reveal the neuroprotective mechanism of Sen, which likely contribute to the development of novel therapies for AD.

Acknowledgments

Author Contributions

All authors made a significant contribution to the conception, study design, execution, and acquisition of data, analysis and interpretation. They participated in drafting, revising or critically reviewing the article. They gave final approval of the version to be published and agreed upon the journal to which the article has been submitted and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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