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Cyanidin suppresses amyloid beta-induced neurotoxicity by inhibiting reactive oxygen species-mediated DNA damage and apoptosis in PC12 cells

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Abstract

Amyloid beta (Aβ)-induced oxidative stress is a major pathologic hallmark of Alzheimer's disease. Cyanidin, a natural flavonoid compound, is neuroprotective against oxidative damage-mediated degeneration. However, its molecular mechanism remains unclear. Here, we investigated the effects of cyanidin pretreatment against Aβ-induced neurotoxicity in PC12 cells, and explored the underlying mechanisms. Cyanidin pretreatment significantly attenuated Aβ-induced cell mortality and morphological changes in PC12 cells. Mechanistically, cyanidin effectively blocked apoptosis induced by Aβ, by restoring the mitochondrial membrane potential *via* upregulation of Bcl-2 protein expression. Moreover, cyanidin markedly protected PC12 cells from Aβ-induced DNA damage by blocking reactive oxide species and superoxide accumulation. These results provide evidence that cyanidin suppresses Aβ-induced cytotoxicity, by preventing oxidative damage mediated by reactive oxide species, which in turn inhibits mitochondrial apoptosis. Our study demonstrates the therapeutic potential of cyanidin in the prevention of oxidative stress-mediated Aβ neurotoxicity.

Key Words: *nerve regeneration; cyanidin; amyloid-beta; oxidative damage; reactive oxide species; apoptosis; neural regeneration*

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Introduction

Alzheimer's disease (AD) is a chronic, age-related, progressive neurodegenerative disease. Deposition of amyloid beta (Aβ) leads to senile plaques, which subject the neuron to oxidative stress and disrupt intracellular connections, resulting in neuronal death. This is considered the main mechanism of AD pathogenesis (Selkoe, 1998; Huang and Jiang, 2009; Eckert et al., 2010; Pagani and Eckert, 2011). Reactive oxygen species (ROS) are small, reactive molecules that play an important role in causing human diseases. Aβ-mediated oxidative stress can attack proteins, membrane lipids and DNA, induce apoptotic cell death, and eventually result in the dysfunction of the neuronal network (Acquaviva et al., 2003). Therefore, blocking Aβ-induced oxidative stress is becoming recognized as an effective strategy by which to combat AD, and potent antioxidant agents show potential in the treatment of neurodegenerative diseases.

Cyanidin, a pigment found in red berries, belongs to the flavonoid family, and has attracted much attention owing to its antioxidant and biological properties (Acquaviva et al., 2003; King et al., 2013). Acquaviva et al. (2003) reported that cyanidin showed marked protective potential against ROS-mediated oxidative damage. Further evidence also suggested that a cyanidin analogue could eliminate overproduced ROS (Chen et al., 2003; Guerra et al., 2005). Cyanidin was also identified as a neuroprotective constituent against oxidative stress (Kim et al., 2005). However, the protective efficiency and mechanism remain to be evaluated in detail. Ye et al. (2010) reported that purple sweet potato anthocyanins could inhibit Aβ-induced cytotoxicity in PC12 cells through inhibition of oxidative stres. Therefore, in the present study, we used the PC12 cell line as an *in vitro* model for evaluating oxidative damage in neurons (Paavlica et al., 2005), to investigate the potential of cyanidin to protect PC12 cells from Aβ-induced neurotoxicity and apoptosis.

Materials and Methods

Cell culture and drug treatment

PC12 cells (ATCC, MD, USA) were maintained in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin and 50 U/mL streptomycin under 5% CO_2 at 37°C. Cells were pre-cultured in a 96-well

plate (1×10^4 cells per well) for 24 hours. $A\beta_{25–35}$ (Sigma, San Francisco, CA, USA) was dissolved in phosphate-buffered saline (PBS) and incubated at 37°C for 3 days prior to treatment. A dose-finding study was performed in which PC12 cells were exposed to cyanidin (5–80 μM) for 24 hours followed by $A\beta_{25–35}$ (5, 10, 20, 40 and 80 μM) for 24 hours. For the protection experiment, PC12 cells were pre-incubated with cyanidin (20, 40, 80 μM; Sigma) for 24 hours followed by $A\beta_{25–35}$ (10 μM) for 24 hours.

MTT assay

Cell viability was assayed using MTT (Fan et al., 2014). After exposure to cyanidin and $A\beta_{25–35}$, 20 μL MTT solution (Sigma; 5 mg/mL) was added to each well for 5 hours at 37°C. The medium was removed, and 100 μL DMSO was added to each well and the plate was incubated for 15 minutes. Color intensity was measured at 570 nm using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as percent MTT reduction, assuming that the absorbance of control cells was 100%. Cells were viewed under a microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

TUNEL/DAPI double staining

A TUNEL-DAPI double staining kit (Beyotime, Beijing, China) was used to measure cell apoptosis, as described previously (Fan et al., 2014). Cells cultured in chamber slides were fixed, permeabilized with 0.1% Triton X-100 in PBS for 2 minutes, treated with TUNEL reaction mixture and terminal deoxynucleotidyl transferase, washed with PBS, and observed under a microscope (Nikon).

Measurement of mitochondrial membrane potential (Δψm)

Δψm was evaluated using JC-1 dye, as described previously (Fan et al., 2014). In brief, PC12 cells were incubated with 80 μM cyanidin for 1 hour, co-incubated with 10 μM $A\beta_{25–35}$ for 2 hours, and then treated with JC-1 probe (10 μg/mL) for 10 minutes. The cells were washed with PBS and visualized using fluorescence microscopy (200× magnification).

Caspase-3 activity

After cyanidin and $A\beta_{25–35}$ treatment, PC12 cells were collected and lysed in buffer, then centrifuged. Protein concentration was measured, and caspase-3 activity was examined as described previously (Fan et al., 2014). Briefly, the caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Cell Signaling Technology, Boston, USA) and 100 μg total protein samples were added to each well of a 96-well plate. The plate was incubated at 37°C in the dark for 2 hours, and caspase-3 activity was measured at excitation and emission wavelengths of 380 nm and 440 nm, respectively.

Detection of ROS and superoxide

Relative levels of ROS were determined using a fluorometric method, the dichloro-dihydro-fluorescein diacetate assay (DCFH-DA; Beyotime). Because free radical generation is an early event, ROS and superoxide were detected at 2 hours, as previously reported (Wang et al., 2015; Fu et al., 2016). Briefly, cells were seeded at 1×10^4 cells/well in a 96-well plate, then incubated for 1 hour with or without 20–80 μM cyanidin, and co-incubated with 10 μM $A\beta_{25–35}$ for 2 hours. DCFH-DA (10 μM) was added to the cells, and ROS generation was monitored at excitation and emission wavelengths of 488 nm and 525 nm, respectively. The superoxide was detected by a mitochondria-targeted fluorogenic dye, MitoSOX (Beyotime).

Western blot assay

Protein expression was evaluated by western blot assay as previously described (Wang et al., 2015; Fu et al., 2016). Briefly, cells were collected and lysed, and protein was quantified using a BCA assay kit. The protein was mixed with buffer, boiled, and 40 μg protein was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane, which was then blocked with 5% non-fat milk (Sigma) for 2 hours and incubated with primary antibodies (1:2,000) for 12 hours at 4°C. Primary antibodies were caspase-3, Bcl-xl, Bcl-2, Bas, Bad, Ser15-p53, total-p53, Ser139-histone and β-actin (all from Cell Signaling Technology). The membrane was then incubated with secondary antibodies (1:2,000) (Cell Signaling Technology) for 2 hours at 37°C. Proteins were visualized on X-ray film and quantified using Quantity-One software (Bio-Rad, Hercules, CA, USA). Target protein expression was normalized to β-actin (a reference control).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± SD. The difference between two groups was analyzed by a two-tailed Student's *t*-test. Differences among three or more groups were analyzed by one-way analysis of variance and multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Cyanidin protected PC12 cells from Aβ-induced cytotoxicity

Cell growth was first assessed in the presence of cyanidin and Aβ alone. The MTT assay showed that PC12 cell viability did not change after cyanidin treatment at concentrations of 5–80 μM (**Figure 1A**). Exposure to Aβ alone (5–80 μM) decreased cell viability in a concentration-dependent manner, and with the greatest toxicity seen at 80 μM (**Figure 1B**). Therefore, we chose a concentration of 10 μM Aβ, which induced approximately 40% cell death, for subsequent experiments. Cell viability was 60.2% after exposure to 10 μM Aβ (**Figure 1C**). However, cyanidin pretreatment effectively blocked the Aβ-induced decrease in PC12 cell viability. At 40 and 80 μM, cyanidin increased cell viability to 79.2% and 94.4%, respectively, from 60.2%. The results demonstrated that cyanidin pretreatment could effectively block Aβ-induced cytotoxicity. Furthermore, cells exposed to Aβ showed apoptotic bodies and were fewer in number compared with control cells. As expected, cyanidin pretreatment markedly improved the morphological changes induced by Aβ (**Figure 1D**). No apoptotic bodies were observed and cell number was markedly greater in the cyanidin pretreatment group

than in the group exposed to Aβ alone. These changes in cell morphology and number further validated the protective potential of cyanidin (**Figure 1D**).

Cyanidin blocked Aβ-induced apoptosis in PC12 cells

The prevention of cyanidin against Aβ-induced apoptosis was first detected using TUNEL and DAPI staining. Apoptotic cells generate many 3′-OH ends, which fluoresce green when subjected to the TUNEL reaction. Thus, green fluorescence represents apoptotic cells. PC12 cells exposed to Aβ alone showed notable apoptotic changes, such as chromatin condensation and nuclear fragmentation (**Figure 2A**). Furthermore, caspase-3 cleavage and activity were also examined. Caspase-3 activity and cleavage were notably elevated after Aβ treatment (**Figure 2B**, **C**; $P < 0.05$). However, cyanidin pretreatment protected PC12 cells from Aβ-induced apoptosis, shown by a decrease in fluorescence intensity, and significantly attenuated caspase-3 activity and cleavage ($P < 0.05$). Collectively, the results affirmed that cyanidin pretreatment markedly suppressed Aβ-induced cytotoxicity by inhibiting Aβ-induced cell apoptosis involving caspase-3 activation.

Cyanidin inhibited Aβ-induced loss of Δψm by regulating Bcl-2

In view of the importance of mitochondria in drug-induced apoptosis, Δψm was examined by JC-1 probe. JC-1 probe can go through the cell mitochondria and show obvious red fluorescence. But, in response to apoptotic stimuli, the probe can transfer into cytoplasm and show green fluorescence. Hence, the fluorescent changes can indirectly reflect the loss of Δψm. The Δψm in PC12 cells was notably decreased after Aβ exposure alone, shown by the enhanced green fluorescence (**Figure 3A**). Cells treated with cyanidin alone caused no change in the Δψm. Pretreatment of cells with cyanidin completely reversed Aβ-induced depletion of Δψm. The result suggests that cyanidin blocked Aβ-induced mitochondrial dysfunction by regulating Δψm. The Bcl-2 family plays a key role in regulating Δψm and launching mitochondria-mediated apoptosis. Bcl-2 family members (including pro-apoptotic and pro-survival members) were examined by western blot assay (**Figure 3B**). Aβ treatment significantly upregulated the expression of Bax and Bad, but downregulated the expression of Bcl-XL and Bcl-2, which indicated that Aβ induced PC12 cell apoptosis by regulation of the Bcl-2 family. However, cyanidin pretreatment markedly blocked Aβ-induced imbalance of Bcl-2 family. Together, these results revealed that cyanidin inhibited Aβ-induced apoptosis and the loss of Δψm by modulation of the Bcl-2 family.

Cyanidin blocked ROS-mediated DNA damage in Aβ-exposed cells

ROS accumulation was measured by DCFH-DA assay. Intracellular ROS was significantly increased to 226.3% of control after treatment with 10 μM Aβ for 1 hour (**Figure 4**). However, pretreatment with cyanidin (20, 40 and 80 μM) caused a marked dose-dependent decline in ROS (to 198.3%, 153.8% and 118.1%, respectively) in cells exposed to Aβ. Furthermore, the superoxide in live cells was also detected by MitoSOX, a mitochondria-targeted red dye. PC12 cells showed notable superoxide overproduction after Aβ treatment in mitochondria, but cyanidin pretreatment markedly blocked this (**Figure 4B**), as evidenced by the weakness of the red fluorescence. These results suggest that cyanidin protects PC12 cells from Aβ-induced cytotoxicity *via* inhibition of ROS and superoxide.

ROS accumulation can damage DNA, even causing apoptotic cell death. Therefore, we examined the effect of cyanidin on Ser15-p53 and Ser139-H2A phosphorylation, both DNA damage markers. Phosphorylated Ser139-H2A and Ser15-p53 were markedly upregulated in PC12 cells exposed to Aβ ($P < 0.05$). As expected, pretreatment with cyanidin completely suppressed phosphorylation of Ser139-H2A and Ser15-p53, to the level observed in control cells ($P < 0.05$), indicating that cyanidin pretreatment prevented Aβ-induced DNA damage. Taken together, the results provide evidence that cyanidin pretreatment markedly attenuates Aβ-induced DNA damage by repressing ROS accumulation.

Discussion

Aβ-induced oxidative stress and redox dysregulation contribute to the etiology and pathology of neurodegenerative diseases (Selkoe, 1998; Huang and Jiang, 2009; Eckert et al., 2010; Pagani and Eckert, 2011). Oxidative stress may damage neuronal cells and is accepted as a major cause of neurotoxicity, and plays an important role in the occurrence and development of human neurodegenerative disorders (Crispo et al., 2010; Tapias et al., 2014). ROS can easily go through the cytoplasmic membrane, bind DNA, and lead to disorders in DNA function (Gao et al., 2013). Antioxidant treatments are beneficial in controlling these diseases (Arai et al., 2007). Therefore, agents with antioxidant activities are usually accepted as chemopreventive agents in epidemiological studies to regulate the progression of oxidative stress-mediated diseases. Increasing evidence indicates that cyanidin, a widely used natural product, exhibits novel anti-oxidant activity and can directly scavenge free radicals. However, its mechanism remains unclear.

Caspases represent the cysteine proteases that have a positive effect in launching cell apoptosis. Caspase-3 can be cleaved and activated in response to apoptosis (Kong et al., 2003; Zhang et al., 2008). Our results indicate that cyanidin blocks PC12 cells from Aβ-induced activation and decreases the expression levels of caspase-3. The changes in caspase-3 activity and caspase-3 expression both validated that cyanidin had the potential to inhibit Aβ-induced apoptosis. ROS is an important small molecule in regulating and inducing cell apoptosis. The balance between ROS accumulation and the cellular defense system ultimately decides the survival or death of the cell (Pabla et al., 2008; Fan et al., 2014). Cyanidin can protect neuronal cells from oxidative damage-induced neurotoxicity by decreasing the generation of ROS (Li et al., 2013; Mokni et al., 2013). In this study, Aβ significantly increased ROS accumulation and activated DNA damage in PC12 cells. However, cyanidin effectively terminated Aβ-induced ROS production and DNA damage. The findings indicated the potential of cyanidin to scavenge free radicals and thus prevent PC12 cells from ROS-mediated oxidative stress and apoptosis.

The antioxidant enzyme system is the endogenous free

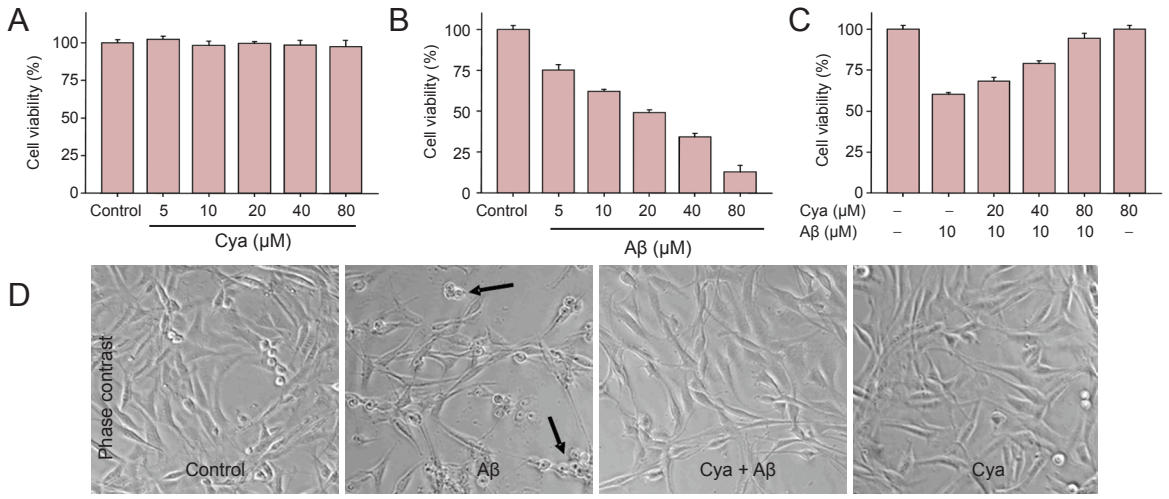

Figure 1 Cyanidin (Cya) suppressed amyloid-beta (Aβ)-induced cytotoxicity in PC12 cells.
(A–C) Effect of Cya on PC12 cell viability (MTT assay). PC12 cells seeded in a 96-well plate were treated with Cya (5–80 μM) for 48 hours (A), Aβ (5–80 μM) for 24 hours (B), or both (Cya 5–80 μM plus Aβ 10 μM; C). Cya protected cells against Aβ-induced cytotoxicity. Cell viability was quantified by MTT assay. Aβ treatment alone (5–80 μM) dose-dependently decreased cell viability ($P < 0.05$), while Cya dose-dependently increased cell viability at 20–80 μM ($P < 0.05$). Multiple comparisons were performed using one-way analysis of variance. Data are presented as the mean ± SD of four independent experiments. (D) Morphological changes in PC12 cells with Cya and Aβ. Arrows, apoptotic bodies.

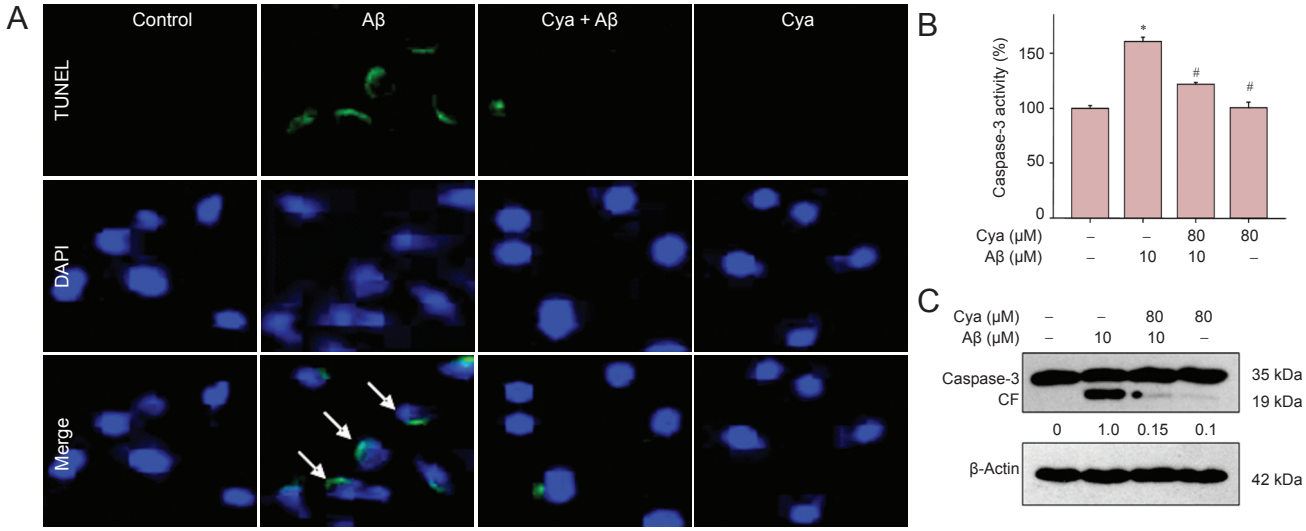

Figure 2 Cyanidin (Cya) prevented amyloid-beta Aβ)-induced apoptosis in PC12 cells.
(A) TUNEL/DAPI double staining. Cells were exposed to 80 μM Cya for 24 hours and/or 10 μM Aβ for 24 hours, and apoptosis was detected by TUNEL/DAPI double staining. Arrows indicate TUNEL-positive cells. (B) Cya blocked Aβ-induced activation of caspase-3, measured by fluorometric assay. *$P < 0.05$, *vs.* control; #$P < 0.05$, *vs.* Aβ treatment. Multiple comparisons were performed using one-way analysis of variance. (C) Caspase-3 expression (western blot). Full length caspase-3 (35 kDa) and the cleavage fraction (CF) of caspase-3 (19 kDa) were examined. Protein expression was measured using Quantity-One software, and all measurements were normalized against β-actin. All experiments were performed three times.

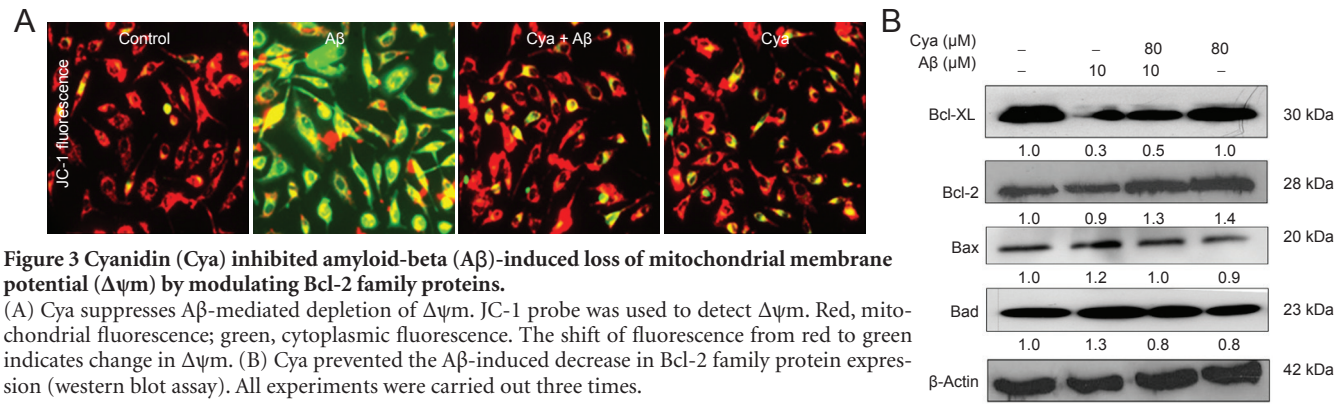

Figure 3 Cyanidin (Cya) inhibited amyloid-beta (Aβ)-induced loss of mitochondrial membrane potential (Δψm) by modulating Bcl-2 family proteins.
(A) Cya suppresses Aβ-mediated depletion of Δψm. JC-1 probe was used to detect Δψm. Red, mitochondrial fluorescence; green, cytoplasmic fluorescence. The shift of fluorescence from red to green indicates change in Δψm. (B) Cya prevented the Aβ-induced decrease in Bcl-2 family protein expression (western blot assay). All experiments were carried out three times.

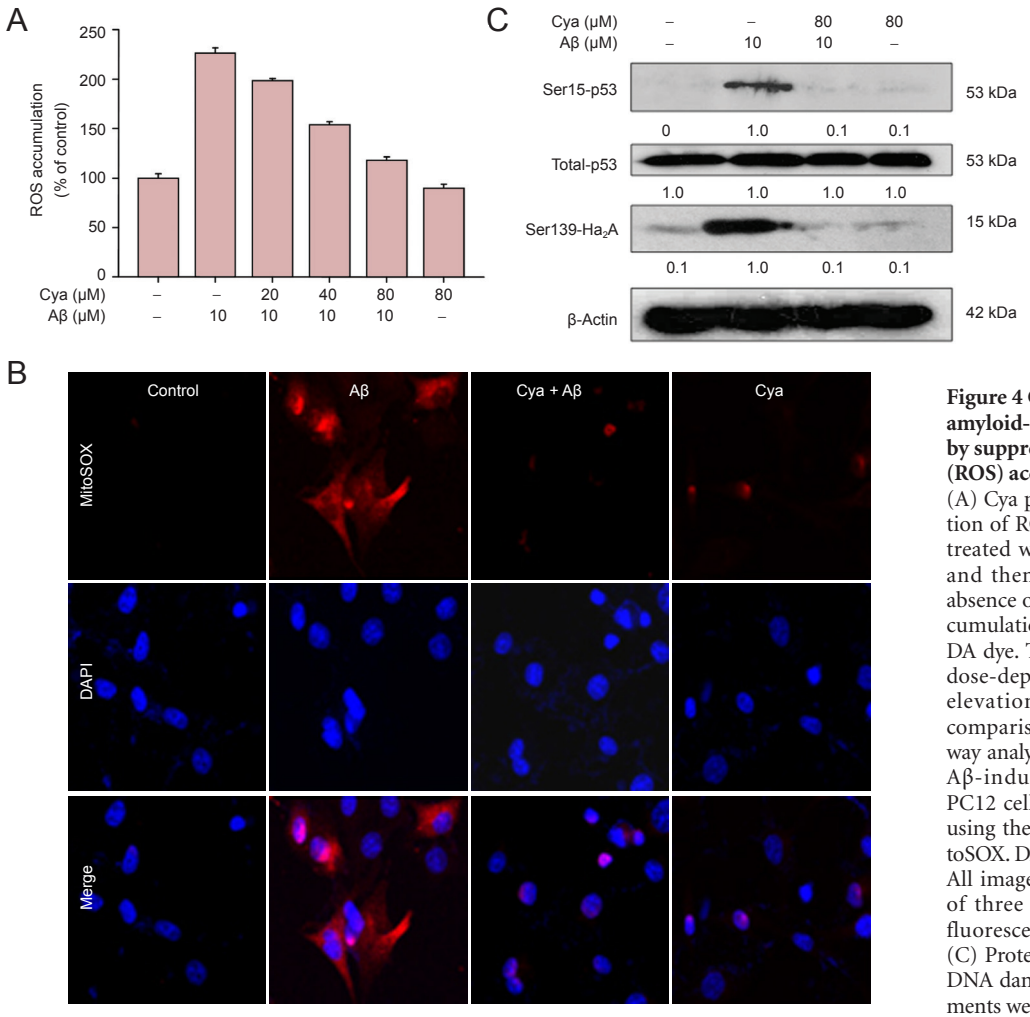

Figure 4 Cyanidin (Cya) prevented amyloid-beta (Aβ)-induced DNA damage by suppressing reactive oxygen species (ROS) accumulation.
(A) Cya prevented Aβ-induced accumulation of ROS in PC12 cells. Cells were pretreated with Cya (20–80 μM) for 1 hour and then incubated in the presence or absence of 10 μM Aβ for 2 hours. ROS accumulation was monitored using DCFH-DA dye. Treatment with Cya at 20–80 μM dose-dependently decreased Aβ-induced elevation of ROS ($P < 0.05$). Multiple comparisons were performed using one-way analysis of variance. (B) Cya inhibited Aβ-induced superoxide production in PC12 cells. The superoxide was observed using the mitochondria-targeted dye, MitoSOX. DAPI was used to stain the nucleus. All images shown here are representative of three independent experiments. Red fluorescence, mitochondrial superoxide. (C) Protection of Cya against Aβ-induced DNA damage (western blot assay). Experiments were conducted three times.

radical scavenger (Mokni et al., 2013). These enzymes can transfer H_2O_2 to water without any toxicity, block the process of lipid peroxide in the body and make the lipid peroxide reduction into a harmless polyol (Kalpana et al., 2007). Therefore, we speculated that the addition of cyanidin may affect the endogenous anti-oxidase system, eliminate the Aβ-induced ROS and ultimately suppress Aβ-induced PC12 cell apoptosis, providing a new idea for development of anti-oxidant drugs in the treatment of neurological disorders.

In conclusion, we evaluated the protective potential of cyanidin against Aβ-induced neurotoxicity in PC12 cells and investigated the underlying mechanism. Our results indicate that cyanidin suppresses Aβ-induced cytotoxicity by suppressing ROS-mediated oxidative damage and inhibiting mitochondria-mediated apoptosis (**Figure 5**).

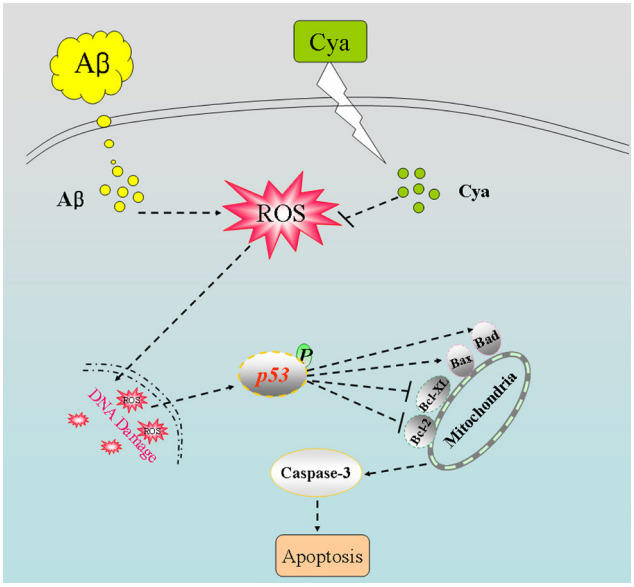

Figure 5 Scheme illustrating potential signaling pathways.
Amyloid-beta (Aβ) directly caused reactive oxygen species (ROS) accumulation, triggered DNA damage and induced PC12 cell apoptosis. However, cyanidin inhibited ROS accumulation, attenuated DNA damage, and eventually reversed Aβ-induced apoptosis in PC12 cells by modulating Bcl-2 family protein expression and restoring the Δψm.

Author contributions: *NCC and ZCZ conceived the whole study. YW, XTF, DWL, KW and YL performed the experiments. XZW, BLS, and XYY analyzed the results. YW and KW wrote the paper. All authors read and approved the final version of this paper for publication.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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