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ORIGINAL PAPER

(2*R*, 3*S*)-pinobanksin-3-cinnamate, a new flavonone from seeds of *Alpinia galanga* willd., presents in vitro neuroprotective effects

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Abstract This study was to investigate the in vitro neuroprotective effects of (2*R*, 3*S*)-pinobanksin-3-cinnamate (PNC) in oxidative stress-induced neurodegeneration models. Pre-incubation of PNC (3-20 μM) with PC12 cells for 24 h significantly decreased the H_2O_2-induced toxicity, shown by elevating the cell viability and decreasing leakage of LDH. Meanwhile, pre-incubation of PNC could attenuate the redox imbalance in PC12 cells through decreasing the levels of MDA and intracellular ROS, enhancing the activity of SOD and the level of GSH. In addition, apoptosis-related biochemical indicators showed that pre-incubation of PNC would elevate the mitochondrial membrane potential, decrease the release of cytochrome-c and formation of DNA fragmentation, and inhibit activities of caspase-3 and caspase-9 in exogenous H_2O_2-treated PC12 cells. These results suggest that PNC may exert its neuroprotective effects on H_2O_2-induced neurotoxicity in PC12 cells, at least partially through counteracting the oxidative stress and preventing apoptosis mediated via mitochondria pathway. PNC had the potential to treat neurodegenerative diseases.

Keywords (2*R*, 3*S*)-pinobanksin-3-cinnamate, Neuroprotective effects, Oxidative stress, H_2O_2, PC12 cell

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The incidence of Parkinson's disease (PD) and Alzheimer's disease (AD) has dramatically increased with the progress of aging of population and brought severe medical and social problems[1,2]. Therefore, there is an urgent need for new drugs for PD and AD therapy, and particularly for drugs with neuroprotective effects.

Studies have revealed that oxidative stress played an important role in the pathogenesis of PD and AD, proved by the decreasing levels of reduced glutathione (GSH) and superoxide dismutase (SOD), increasing levels of reactive oxygen species (ROS), in PD and AD patients and animal models[3,4]. Oxidative stress can be induced by in vitro administration of cytotoxic agents such as 6-hydroxydopamine[5], amyloid β[6] and H_2O_2[7,8]. The latter is a well-known model of oxidative stress in neuronal cell cultures. Exposure of neurons to H_2O_2 will result in the activation of a cascade of intracellular toxic events like lipid peroxidation, liberating free radicals, which cause alteration of the mitochondrial membrane permeability, leading to neuronal cell death[9].

Supplementation of exogenous antioxidants has their effects in curtailing the oxidative damage to cellular macromolecules. One category of such antioxidants is the flavonoids, which have received more and more attentions due to their biological activities, including anti-oxidative, radical scavenging and anti-inflammatory effects, most interestingly in attenuating the redox imbalance and improving neuro-cognitive performance[10,11]. (2*R*, 3*S*)-pinobanksin-3-cinnamate (PNC, Figure 1) was a new flavonone isolated from the seed of *Alpinia galanga* Willd[12], which mainly distributes in the south China and has been used in traditional medicine systems for its anti-oxidant activities[13]. Additionally, ethanol extract of *Alpinia galanga* Willd was reported to enhance the cognitive function by decreasing the level of acetylcholinesterase (AChE) and reduce

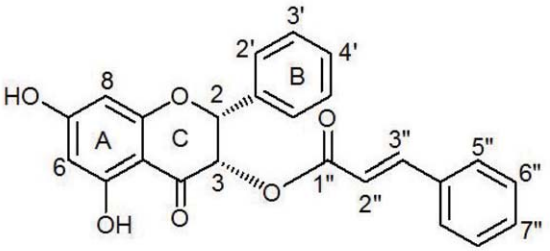

Figure 1. Structure of (2*R*, 3*S*)-pinobanksin-3-cinnamate (PNC).

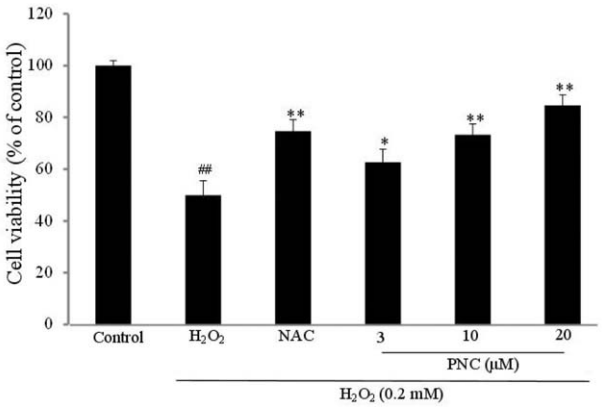

Figure 2. Effect of PNC on the cell viability of H_2O_2-damaged PC12 cells by MTT assay. Pre-incubation with PNC or NAC (positive control) for 24 h protected PC12 cells from H_2O_2 toxicity (0.2 mM, 24 h). NAC: N-acetyl-L-cysteine. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

the generation of free radicals[14]. Other fractions (n-hexane, chloroform and ethyl acetate) of *Alpinia galanga* Willd were also exerted the neuroprotective effect in Alzheimer's type of amnesia in mice induced by amyloid beta[15].

This study was to investigate the neuroprotective effects of PNC in oxidative stress-induced neurodegenerative models. Furthermore, the possible action mechanisms of neuroprotective effects were explored.

Effects of PNC on H_2O_2-induced cytotoxicity

PC12 cell viability was reduced after treatment with 0.2 mM H_2O_2 for 24 h, as shown by Figure 2. The pre-treatment with PNC (3, 10, 20 μM) and NAC (20 μM) for 24 h significantly increased the cell viability in a concentration-dependent manner, comparing with H_2O_2 model group ($P<0.05$).

LDH leakage assay

To confirm the neuroprotective effect of PNC on H_2O_2-damaged PC12 cells, LDH leakage was measured. Figure 3 showed that treatment with 0.2 mM H_2O_2 for 24 h significantly increased the LDH leakage from PC12 cells. However, the pre-treatment with PNC (3, 10, 20 μM) for 24 h significantly decreased the H_2O_2-induced LDH leakage ($P<0.05$).

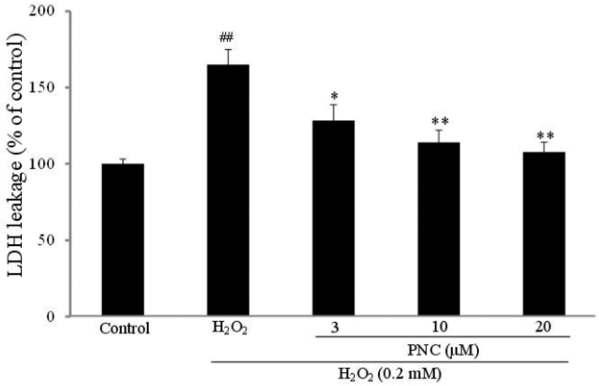

Figure 3. Effect of PNC on the LDH leakage in H_2O_2-damaged PC12 cells. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

Measurement of MDA, ROS, SOD and GSH levels

The PC12 cell oxidative stress was assessed by MDA level, intracellular ROS level, the activity of SOD and GSH. Figure 4 showed that treatment of PC12 cells with H_2O_2 significantly increased MDA and ROS level, while decreased the activity of SOD and GSH. The pre-treatment with PNC (3, 10, 20 μM) for 24 h significantly reversed the oxidative stress in PC12 cells, comparing with H_2O_2 model group ($P<0.05$).

Change of mitochondrial membrane potential

Figure 5 showed that treatment with 0.2 mM H_2O_2 for 24 h led to an obvious decrease of MMP. However, the depolarization induced by H_2O_2 was attenuated by pre-treatment with PNC (3, 10, 20 μM) for 24 h ($P<0.05$).

Measurement of cytochrome-c

Figure 6 showed that treatment of PC12 cells with 0.2 mM H_2O_2 for 24 h significantly increased the cytochrome-c releasing into cytoplasm. However, the pre-treatment with PNC (3, 10, 20 μM) for 24 h significantly decreased cytochrome-c level in cytoplasm of PC12 cells ($P<0.05$).

Quantification of DNA fragmentation

Figure 7 showed that exposure to 0.2 mM H_2O_2 for 24 h significantly increased the DNA fragmentation in PC12 cells. The pre-treatment with PNC (3, 10, 20

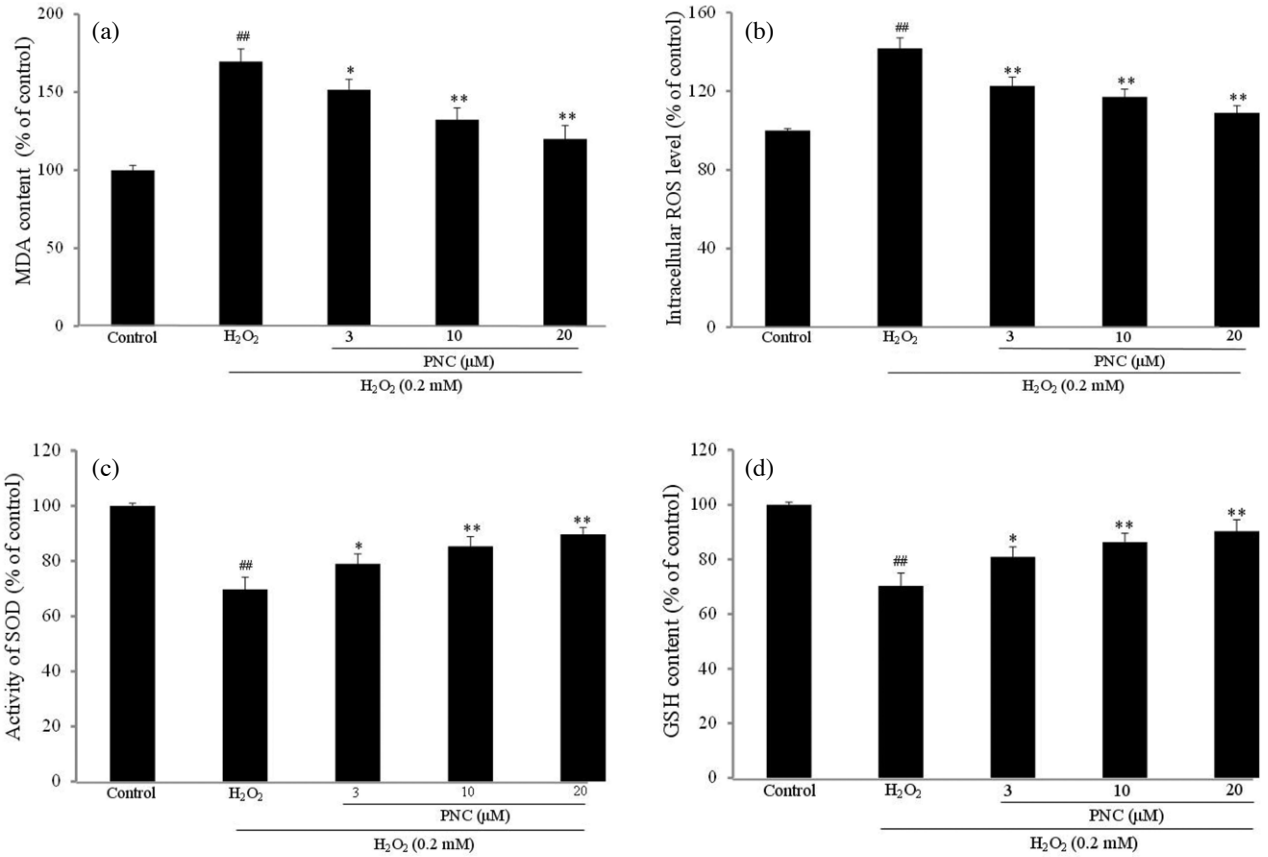

Figure 4. Effect of PNC on H_2O_2-induced oxidative stress in PC12 cells. Oxidative stress was evaluated by examining MDA content (a), intracellular ROS level (b), SOD activity (c), and GSH content (d). Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

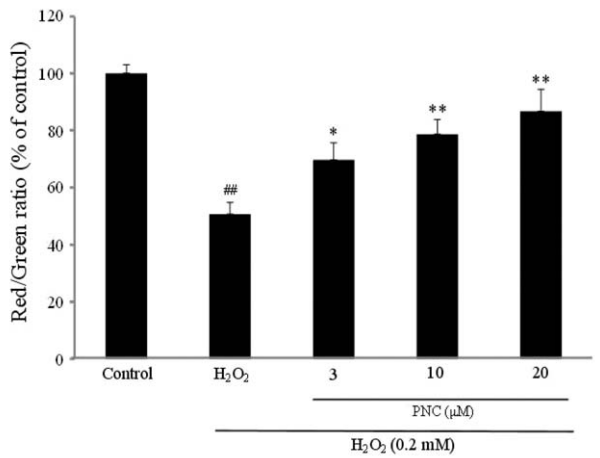

Figure 5. Effect of PNC on mitochondrial membrane potential in H_2O_2-damaged PC12 cells assayed by JC-1. JC-1 is sensitive to MMP, and the changes in the ratio between aggregate (red) and monomer (green) fluorescence can provide information regarding the MMP. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

μM) for 24 h significantly decreased the H_2O_2-induced DNA fragmentation ($P<0.05$).

Activities of caspase-3 and caspase-9

As shown in Figure 8, after treatment of PC12 cells with 0.2 mM H_2O_2 for 24 h, activities of caspase-3 and caspase-9 increased significantly, comparing with the control group. However, after cells were incubated with PNC (3, 10, 20 μM) for 24 h prior to exposure to H_2O_2, caspase activities were significantly decreased ($P<0.05$).

Discussion

Oxidative stress, mainly mediated by overproduction of ROS, can affect various biological macromolecules including lipids, proteins, and DNA, to cause lipid peroxidation, protein denaturation, inactivation of enzymes, nucleic acid and DNA damage, mitochondrial dysfunction, thereby impairing cellular function

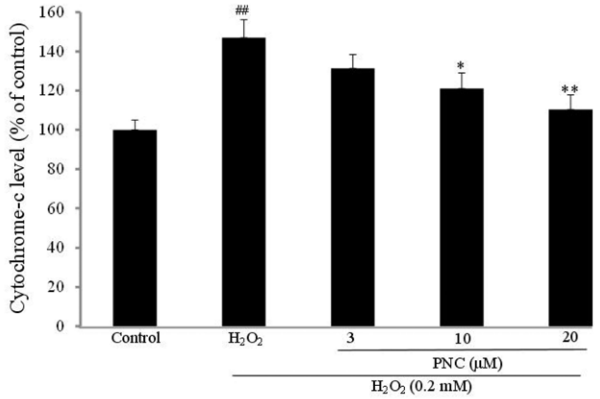

Figure 6. Effect of PNC on the cytochrome-c releasing in H_2O_2-damaged PC12 cells. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

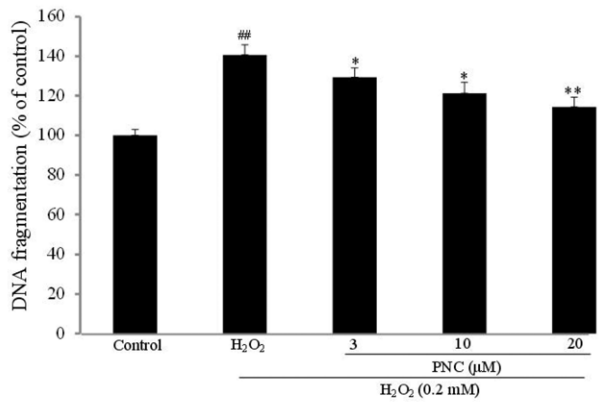

Figure 7. Effect of PNC on DNA fragmentation of H_2O_2-damaged PC12 cells. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

and integrity[16-18]. This type of damage has been considered as a major cause of cellular injuries and neuronal damages in some neurodegenerative disorders such as PD and AD[19-22]. Therefore, the imbalance between the intracellular oxidative and anti-oxidative defense systems including SOD and GSH, requires the supplement of external antioxidants to eliminate ROS as a potential therapeutics in some neurodegenerative diseases.

ROS generation and the resultant oxidative stress have been implicated widely in the mechanism of H_2O_2-induced cell death. MDA is a by-product of lipid peroxidation produced under oxidative stress and is well-known as a widely-used marker for oxidative damage of plasma membrane and resultant thiobarbituric acid reactive substances, which are proportional to lipid peroxidation and oxidant stress[23]. It is well-known that flavonoids have potent anti-oxidative and free radical-scavenging effects[24-28]. In this study, we demonstrated that PNC dramatically elevated H_2O_2-damaged cell viability. The protective effect of PNC might be attributed to the presence of two hydroxyl groups with reductive activity, bringing about its powerful antioxidant action and effective free radical-scavenging ability, as evidenced by the markedly reduced MDA and ROS levels as well as the enhanced SOD and GSH activity.

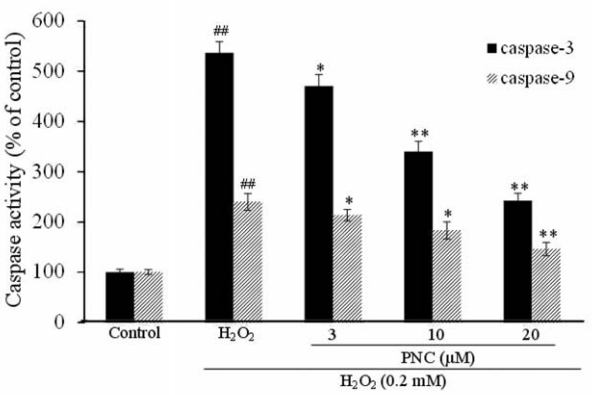

Figure 8. Effect of PNC on caspase activity of H_2O_2-damaged PC12 cells. Values were expressed as mean ± SD (n=6). $^{\#\#}P<0.01$ *vs* control group; $^{*}P<0.05$, $^{**}P<0.01$ *vs* H_2O_2 group.

Now it is clear that mitochondria play a pivotal role in the cell death regulation process. Many genes and proteins can influence or determine the progression of apoptosis along the mitochondrial pathway[29,30]. Overproduction of ROS would cause disturbance in the mitochondria[31] and the change of mitochondrial membrane potential could induce the release of cytochrome-c from the mitochondrial to nucleus, which could activate caspase-related apoptosis protein and facilitate the formation of apoptosome complex, leading to chromatin condensation and DNA cleavage. DNA fragmentation has been considered as a biochemical hallmark and widely used as apoptosis index[32-35]. Therefore, the depolarization of MMP and the release of cytochrome-c from mitochondria in PC12 cells were detected to illustrate the onset of apoptosis[36]. In the present study, we found that treatment of PC12 cells with H_2O_2 led to cell injury and DNA fragmentation, accompanied by MMP depolarization and cytochrome-c release from mitochondria. However, after pre-incubation with PNC, the elevation of cytochrome-c release and the loss of MMP were ameliorated to impede the apoptotic protein activation.

Caspases play an important role in the mitochondria-dependent apoptotic process. A variety of stimuli trig-

gers the mitochondrial permeability transition and the release of cytochrome-c, and then activation of caspases[37]. Caspase-3 acts as an apoptotic executor and activates DNA fragmentation factor, which in turn activates endonucleases to cleave nuclear DNA and ultimately leads to cell death. So inhibition of caspsase-3 has a promising effect in attenuating apoptosis[38]. In addition, caspase-9 was generally activated following the disruption of the outer mitochondrial membrane, which caused a collapse of membrane potential and a change of permeability[39]. Previous study showed that the cytochrom-c released from mitochondria combined with caspase-9 precursor and resultantly led to activation of caspase-9 activity[40]. Our results implied that exposure of PC12 cells to H_2O_2 significantly increased activities of caspase-3 and caspase-9. However, preincubation with PNC reversed the elevation of caspase-3 and caspase-9 activities.

In summary, this study demonstrated that PNC increased the viability of PC12 cells, attenuated the imbalance of redox and inhibited cellular apoptosis in H_2O_2-induced oxidative stress model. PNC effectively protected PC12 cells against H_2O_2-induced oxidative injury, most possibly through mitochondria pathway, closely related to its antioxidant activities. These exciting results warrant further study as the potential agent for the treatment of neurodegenerative disease.

Materials & Methods

Cell line, chemicals and reagents

Differentiated PC12 cell line was obtained from Shanghai Cell bank (Shanghai, China). N-acetyl-L-cysteine (NAC) and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium-bromid (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). LDH, MDA, SOD and GSH assay kits were purchased from Jiacheng Institute of Biological Engineering (Nanjing, China). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (CA, USA). Cytochrome-c enzyme-linked immunosorbent assay kit was from R&D systems (Minneapolis, MN, USA). Cell death Detection ELISA^{plus} kit was from Roche Applied Sciences (Switzerland). Caspase-3 and caspase-9 activity assay kits were purchased from Kaiji Institute of Biological Engineering (Nanjing, China). All solvents and other chemicals used in this study were of analytical grade and purchased from Sinopharm chemical reagent Co. Ltd. (Shanghai, China). PNC (purity $>98\%$) was isolated and identified by Department of Chemistry of Natural Product, Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China).

Cell culture and treatment

Differentiated PC12 cells were used in the experiments *in vitro*. PC12 cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/mL Penicillin, and 100 μg/mL Streptomycin, at 37°C in humidified 95% air/5% CO_2. PC12 cells were treated by the following process: cells were incubated for 24 h with PNC or NAC (positive control), respectively; cells treated by the same solvent alone as the negative control. Consequently, PC12 cells were treated by 0.2 mM H_2O_2 for 24 h.

Cell viability measurement

Cell viability was determined by MTT assay. PC 12 cells were plated at a density of 1×10^4/100 μL in 96-well plates and grown overnight before treatment. After treatment, 10 μL 0.5 mg/mL MTT solution was added in each well and incubated for another 4 h at 37°C. Culture medium was removed and the formazan crystals were solubilized with 100 μL DMSO. The absorbance was measured at 570 nm by a microplate reader (BIOTEK, USA). Cell viability was expressed as the percentage of the negative control, which was set to 100%.

LDH leakage assay

The LDH leakage was measured by a LDH assay kit. Briefly, after treatment, the medium was collected and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was used to measure the activity of LDH according to the manufacturer's instructions. The absorbance was measured by a microplate reader at 490 nm. The LDH leakage content was expressed as the percentage of the negative control, which was set to 100%.

Measurement of MDA level, activity of SOD and GSH

The MDA level, activity of SOD and GSH were measured by the corresponding assay kit. Briefly, after treatment, cells were washed twice in ice-cold PBS and homogenized. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was used for MDA, SOD and GSH assay according to the manufacture's instructions. The levels of MDA, SOD and GSH were expressed as a percentage of the negative control, which was set to 100%.

Measurement of ROS level

Intracellular ROS were measured by oxidation-sensitive fluoroprobe DCFH-DA. Briefly, after treatment, cells were washed twice in ice-cold PBS and incubated with DCFH-DA at the final concentration of 10 μM for 30 min at 37°C. Cells were then washed twice with PBS and the fluorescence intensity was measured by a fluorescent spectrophotometer (Thermo Scientific, USA) at 488/525 nm. The levels of intracellular ROS were expressed as a percentage of the negative control, which was set to 100%.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was measured by the fluorescent probe JC-1. The ratio between aggregate (red) and monomer (green) fluorescence can reflect the MMP. After treatment, PC12 cells were washed twice with PBS and loaded with JC-1 for 15 min at 37°C in the dark. After two more rinses with Hank's solution, the fluorescence intensity of the red/green was determined on a fluorescence microplate reader (TECAN Polarion, UK) at an excitation of 490 nm and emission of 530 nm (green fluorescent monomers) and 590 nm (red fluorescent aggregates) respectively. The change of MMP was expressed as a percentage of the negative control, which was set to 100%.

Cytochrome-c assay

The cytosolic cytochrome-c was measured by the assay kit. After treatment, PC12 cells were washed twice with PBS and fractionated. The cytochrome-c was determined according to the manufacturer's instructions. After reaction, the optical density was measured by a microplate reader at 490 nm. The level of cytochrome-c was expressed as a percentage of the negative control, which was set to 100%.

Measurement of DNA fragmentation

Quantification of DNA fragmentation was measured by Cell Death Detection ELISA^{plus} kit. Briefly, after treatment, PC12 cells were washed twice with PBS and lyzed for 30 min. After a centrifugation at 1,000 rpm for 10 min at 4°C, 20 μL supernatant was transferred to a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. The peroxidase amount in the immunocomplex was quantified by adding 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. The absorbance of the reaction mixture was measured by a microplate reader at 405 nm. The extent of DNA fragmentation was expressed as a percentage of the negative control, which was set to 100%.

Measurement of caspase activity

The caspase activity was measured by the assay kit according to the manufacturer's instructions. After treatment, PC12 cells were washed twice with PBS and lyzed with a buffer consisting of 1 mM EDTA-Na, 1 mM EGTA-Na, 2 mM $MgCl_2$, 25 mM HEPES (pH 7.5), 0.1% CHAPS, 100 mM PMSF, 2 mM DTT and 1 : 100 broad spectrum protease inhibitor. Cell extracts were centrifuged at 20,000 rpm for 10 min at 4°C and aliquots of supernatants containing 25 mg protein were added to a reaction buffer (25 mM HEPES, pH 7.5) supplemented with 0.1% CHAPS, 5 mM DTT and 100 mM PMSF. The reactions were initiated after addition of the following fluorescent substrates (50 mM final concentration): Ac-DEVD-Amc for caspase-3 activity, Ac-LEDH-Afc for caspase-9 activity. After 2 h of incubation at 37°C, the cleavage of the substrates was measured (Amc: 390/475 nm; Afc: 400/505 nm) by a microplate reader. The activity of caspase was expressed as a percentage of the negative control, which was set to 100%.

Statistical analysis

Values were presented as mean ± SD and statistically analyzed by one-way analysis of variance (ANOVA) using the Sigma Stat statistical software (SPSS Inc., Chicago, IL). Differences were considered as significant at $P < 0.05$.

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Conflict of Interest The authors declare no conflict of interest.

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