ORIGINAL ARTICLE

Effects of Senegenin against Hypoxia/Reoxygenation-Induced Injury in PC12 Cells*

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ABSTRACT **Objective**: To investigate the effect and the potential mechanism of Senegenin (Sen) against injury induced by hypoxia/reoxygenation (H/R) in highly differentiated PC12 cells. **Methods**: The cultured PC12 cells were treated with H/R in the presence or absence of Sen (60 μmol/L). Four groups were included in the experiment: control group, H/R group, H/R+Sen group and Sen group. Cell viability of each group and the level of lactate dehydrogenase (LDH) in culture medium were detected for the pharmacological effect of Sen. Hoechst 33258 staining and annexin V/propidium iodide double staining were used to analyze the apoptosis rate. Moreover, mitochondrial membrane potential (△Ψm), reactive oxygen species (ROS) and intracellular free calcium ($[Ca^{2+}]i$) were measured by fluorescent staining and flow cytometry. Cleaved caspase-3 and activity of NADPH oxidase (NOX) were determined by colorimetric protease assay and enzyme linked immunosorbent assay, respectively. **Results**: Sen significantly elevated cell viability ($P<0.05$), decreased the leakage of LDH ($P<0.05$) and apoptosis rate ($P<0.05$) in H/R-injured PC12 cells. Sen maintained the value of △Ψm ($P<0.05$) and suppressed the activity of caspase-3 ($P<0.05$). Moreover, Sen reduced ROS accumulation ($P<0.05$) and $[Ca^{2+}]i$ increment ($P<0.05$) by inhibiting the activity of NOX ($P<0.05$). **Conclusion**: Sen may exert cytoprotection against H/R injury by decreasing the levels of intracellular ROS and $[Ca^{2+}]_i$, thereby suppressing the mitochondrial pathway of cellular apoptosis.

KEYWORDS senegenin, hypoxia/reoxygenation, PC12 cells, reactive oxygen species, NAPDH oxidase

Ischemic cerebrovascular disease is the leading cause of morbidity and mortality worldwide. Reperfusion of blood flow is needed after cerebral ischemia. However, clinical observations and animal experiments showed that reperfusion itself is a source of noxious effects (calcium overload, free radicals production and mitochondrion alteration) that paradoxically aggravate cell damage and even cell death, especially in the case of a prolonged ischemia. The mechanisms underlying the pathogenesis of ischemia/reperfusion injury are complex and not completely understood. Generally accepted mechanisms include free radical damage,[1] calcium overloading,[2] inflammation mediated by leukocytes,[3] excessive release of excitatory amino acids and apoptosis.[4,5] These mechanisms have been used to help identify neuroprotective agents to treat ischemic stroke. However, effective therapy has not yet been achieved.

Chinese medicine (CM), well-known for its multi-pathway and multi-target effects, has recently been used for the treatment of cerebral ischemia/reperfusion (I/R) injury. Senegenin (Sen) is the major and most active ingredient of *Radix Polygala*, a traditionally medical herb that has been widely used in China. Chen, et al[6] found that Sen could inhibit the release of lactate dehydrogenase (LDH), maintain the normal morphology of neuron synaptic and adherent status, and promote cell viability, thus protect against cytotoxicity induced by $A\beta_{1-40}$ in primary cultured

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cortical neurons. Sun, et al[7] revealed that Sen could reduce the level of malondialdehyde (MDA), and increase superoxide dismutase (SOD) activity, thus protect against H_2O_2-induced injury in PC12 cells. In addition, Sen was shown to attenuate hepatic ischemia-reperfusion inducing cognitive dysfunction by increasing hippocampal NR2B expression in rats.[8] Moreover, recent studies addressed that Sen had neurotrophic effect on cultures of newborn cortical neurons[9] and promoted proliferation of human neural progenitor cells by upregulating phosphorylation of extracellular signal-regulated kinase *in vitro*.[10] In a word, Sen displayed anti-apoptotic and anti-oxidative activity, and possessed neuroprotective and neuroregenerative effects.

Despite of many evidences showing that Sen could improve memory and intelligence,[11] and enhance cognitive functions in elderly individuals,[12] few studies with respect to the effect of Sen on cerebral ischemia/reperfusion injury have been reported.

As reported in previous studies, mitochondria-dependent apoptotic pathway can be triggered by hypoxia-reoxygenation (H/R)-mediated injury.[13] Meanwhile, the loss of mitochondrial membrane potential ($\triangle\Psi m$) has been proved to be hallmarks of mitochondria-dependent apoptosis.[14] Those studies also implied that caspase-3, whose activation is involved in mitochondria-dependent apoptotic pathway, is the final executor of apoptosis. Reactive oxygen species (ROS) plays a vital role in neuronal cell apoptosis mediated by ischemia/reperfusion injury.[15] Mitochondria are deemed as a sensor of ROS. It is well-known that overproduction of ROS can cause the increasing of membrane permeability and then lead to intracellular free calcium $[Ca^{2+}]_i$ overload. In addition, there is a positive feed-back loop of intracellular Ca^{2+} overload and mitochondrial ROS generation. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) also contributes significantly to ROS generation following ischemic reperfusion besides mitochondrial ROS generation.[16]

It is indicated in our preliminary result that H/R-induced injury in PC12 cells was attenuated by Sen and this protective effect was associated with anti-apoptotic effect of Sen. Thereupon, several key points including $\triangle\Psi m$, caspase-3, intracellular Ca^{2+}, ROS and NADPH oxidase (NOX) which are essential in apoptotic signaling pathway were selected to probe in the following study. It is backward induction that we design to get access to the protective mechanism of Sen. It was implied in the final results that the neuroprotective effect of Sen was associated with the maintenance of $\triangle\Psi m$ and preventing the activation of caspase-3 by attenuating intracellular ROS accumulation and calcium overload. In the present study, the question whether Sen could protect against H/R-induced injury in PC12 cells was examined.

METHODS

Chemicals

Sen (MR537.1 of purity>98.5%) was purchased from Guangzhou Institute for Drug Control (China, batch No. 111572-200702) and dissolved in dimethylsulfoxide (DMSO) at 80 mmol/L. It was then diluted to 15, 30, 60 and 80 μmol/L for experiments.

Cell Model of H/R *in Vitro*

In vitro model of H/R employed in this study was similar to what was described previously.[17,18] Briefly, 80% confluent PC12 cells (purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China) in glucose-free medium (137 NaCl; 12 KCl; 0.9 $CaCl_2 \cdot 2H_2O$; 0.49 $MgCl_2$; 5 HEPES; 20 $NaHCO_3$, in mmol/L) in 6-well culture plates were exposed to anoxia for 4 h in MGC AnaeroPack (Mitsubishi Gas Chemical Company, Inc., Japan). The cells were subjected to reoxygenation with a shift of the medium into DMEM without fetal bovine serum (FBS) followed by incubation under normoxia for 2 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide Assay

PC12 cells were seed in 96-well plates (1×10^4 cells/well). Sen was added to the medium (final concentration 15, 30, 60, 80 μmol/L) with or without H/R treatment. Subsequently, 20 μL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were added to each well and cells were incubated at 37 ℃, 5% CO_2 for 4 h. Then the medium of each well was replaced by 150 μL DMSO. The absorbance was measured at 490 nm and the relative ratio of cell viability was calculated.

LDH Assay

PC12 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and they were incubated

at 37 ℃, 5% CO_2 for 24 h. Then, Sen was added to the medium to obtain final concentration of 15, 30, 60 μmol/L respectively with or without H/R treatment. Six hours later, the resulting supernatant was collected for the quantity of LDH assay by an automatic biochemical analyzer (Hitachi Company, Japan).

Hoechst 33258 Staining

Nuclei were visualized by staining cells after fixation. Treated cells were fixed in 4% paraformaldehyde for 10 min and then incubated with Hoechst dye 33258 at 1 mg/mL in phosphate-buffered saline (PBS) without paraformaldehyde for 5 min at room temperature in darkness. Subsequently, cells were washed thrice with PBS and imaged by a fluorescent microscope (excitation 350 nm; emission 460 nm, type Ⅸ-71, Olympus Company, Japan). Apoptotic cells were scored when chromatin condensation or fragmentation emerged.

Quantitative Analysis of Apoptosis Using Flow Cytometer

Treated cells were washed twice with PBS (2,000 r/min, 5 min) and were reset at a density of 5×10^5/mL. Then, the cells were resuspended in 500 μL binding buffer, and 5 μL annexin V-FITC along with 5 μL propidium iodide (PI) was added at room temperature in darkness for 5–15 min. All samples were then analyzed under a flow cytometer (FACSAria, Becton, Dickinson and Company, America) within 1 h (excitation 488 nm; emission 530 nm).

Measurement of Mitochondrial Membrane Potential

Treated cells were washed with PBS, and then 1 mL DMEM and 1 mL JC-1-fluorescent dye were added to cells and rocked gently. Cells were incubated at 37 ℃ and 5% CO_2 for 20 min. Meanwhile, the JC-1 buffer (1×) was prepared on ice. After incubation, the cultured medium was removed and cells were washed twice with JC-1 buffer (1×). For fluorescent microscope analysis, 2 mL DMEM was added to resuspend cells. For flow cytometer analysis, cells were resuspended in JC-1 buffer. The monomeric form was detected at excitation 490 nm, emission 530 nm while dimeric form at excitation 525 nm, emission 590 nm.

Caspase-3 Activity Assay

Cleaved caspase-3 was determined by colorimetric protease assay kit. The assay was performed according to the manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd., China). In brief, treated cells was washed twice with PBS. Then chilled cell lysis buffer was added to cells for 20–60 min at 4 ℃, and the resulting supernatant was collected via centrifuging for 1 min in a microcentrifuge (10,000×*g*). The resulting supernatant was transferred to a fresh tube and the protein concentration was measured. Supernatant containing 100–200 μg protein was incubated at 37 ℃, for 4 h in darkness after the addition of 2× reaction buffer (containing dithiothreitol) and caspase-3 substrate. Finally, the absorbance was measured at 405 nm and the activity of caspase-3 was calculated.

Measurement of Intracellular ROS

2'7'-Dichlorofluorescin diacetate (DCFH-DA) was diluted with FBS-free DMEM to a concentration of 10 μmol/L. Treated cells were resuspended with diluted DCFH-DA and incubated at 37 ℃, and 5% CO_2 for 20 min. Then, cells were washed thrice with FBS-free DMEM. All samples were analyzed under a flow cytometer (excitation 488 nm; emission 525 nm).

Measurement of Intracellular Calcium Concentration

The intracellular calcium levels were determined by fluorescent agent Fluo-3AM. Fluo-3AM ester is a calcium indicator that is widely used for cell-based functional assay. It is essentially non-fluorescence unless bound to calcium, and this fluorescence increases with rising calcium levels. Briefly, at the end of cell treatment, the supernatant was discarded and the cells were incubated with complete medium containing 5 μmol/L Fluo-3AM at 37 ℃, and 5% CO_2 for 30 min. Then, the cells were washed with PBS and resuspended with PBS for detection by a flow cytometer (excitation 488 nm; emission 525 nm).

NADPH Oxidase Activity Assay

The activity of NADPH oxidase was measured by NADPH oxidase elisa kit (RD-corporation, USA). The samples were simply added to pre-coated microtiter plate wells with NADPH oxidase antibody which was labeled with horse radish peroxidase (HRP) and they were incubated for about 30min at 37 ℃. Tetramethylbenzidine (TMB) substrate solution was added when it had been completely washed, and it turned blue when HRP enzyme-catalyzed. The reaction was terminated and the color

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shifted from blue to yellow when a sulphuric acid solution was added. This color transform is measured spectrophotometrically at a wavelength of 450 nm. The concentration of NADPH oxidase in the samples was then determined by comparing the optical density (OD) value of the samples with the standard curve.

Statistical Analysis

All values were expressed as mean ± standard deviation ($\bar{x} \pm s$), significant differences between the groups were evaluated via one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. Two-group comparisons were performed using an LSD test or Tamhane's T2 test. $P<0.05$ was considered to be statistically significant.

RESULTS

Determination of Working Concentration of Sen

To determine the working concentration of Sen, PC12 cells were treated with different concentrations of Sen (15, 30, 60, 80 μmol/L). Sen (15, 30, 60 μmol/L) did not affect cell viability under incubation for 6 h ($P>0.05$), while a higher concentration (80 μmol/L) reduced cell viability significantly ($P<0.05$). The concentrations of Sen were selected at 15, 30, 60 mol/L in this experiment (Figure 1).

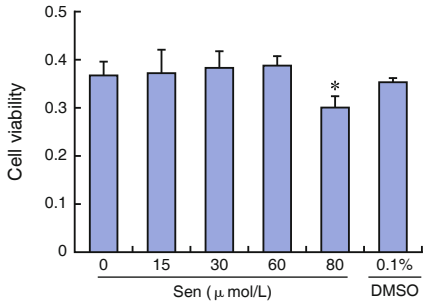

Figure 1. Effect of Sen on PC12 Cells by MTT Assay

Notes: PC12 cells were treated with different concentrations of Sen for 6 h, and the cell viability was evaluated by MTT assay. Values of $\bar{x} \pm s$ were from three independent experiments. $^{*}P<0.05$, compared with the control group

Effect of Sen against H/R-Induced Injury in PC12 Cells

The cytoprotective effect of Sen was detected by MTT assay and LDH activities in PC12 cell injured by H/R. As shown in Figure 2A, the viability of PC12 cells in H/R group was significantly decreased compared to control group. Interestingly, cell viability in H/R+Sen (15, 30, 60 μmol/L) increased to 32.25% ± 5.17%, 37.52% ± 3.59% and 41.88% ± 2.90%, respectively. When PC12 cells were exposed to H/R injury, the leakage of LDH was significantly increased ($P<0.05$). In contrast, when the cells were treated with different concentrations of Sen (15, 30, 60 μmol/L), the LDH leakage was decreased as the dosage increased gradually (Figure 2B).

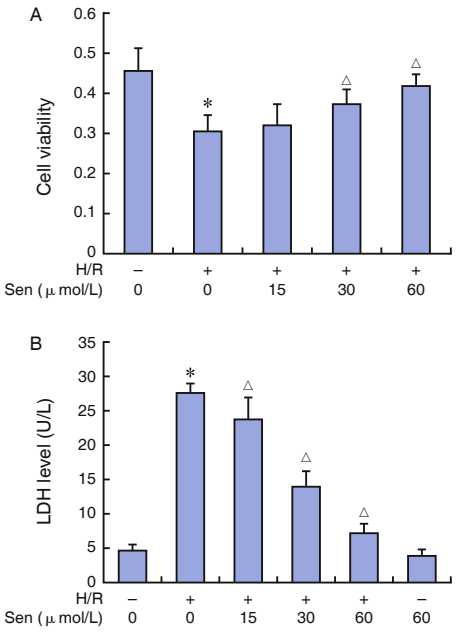

Figure 2. Effect of Sen against H/R-Induced Injury in PC12 Cells

Notes: A: Sen prevented H/R-induced decrease in PC12 cell viability; B: Sen attenuated H/R-induced LDH leakage in PC12 cells. Values are $\bar{x} \pm s$ from three independent experiments. $^{*}P<0.05$, compared with the control group; $^{\triangle}P<0.05$, compared with the H/R group

Effect of Sen against H/R-Induced Apoptosis Detected by Hoechst33258 Staining

Hoechst33258 staining showed that the nuclei of normal cells appeared to be of similar size and regular conformation, while the number of cells with bright blue fluorescence condensed nuclei and chromatin fragmentation were increased in the H/R group. Some cells showed pyknosis and karyorrhexis with others showing typical apoptotic bodies. The apoptosis was notably inhibited after treatment of Sen. As the concentration of Sen increased, the number of normal cells rose gradually, the morphology of cells was improved, and the phenomena of pyknosis and karyorrhexis were gradually decreased (Figure 3).

Effect of Sen against H/R-Induced Apoptosis in PC12 Cells Detected by Flow Cytometer

To quantify the effect of Sen on H/R-induced PC12 cell apoptosis, the percentage of apoptotic cells was detected by annexin V-FITC and PI double

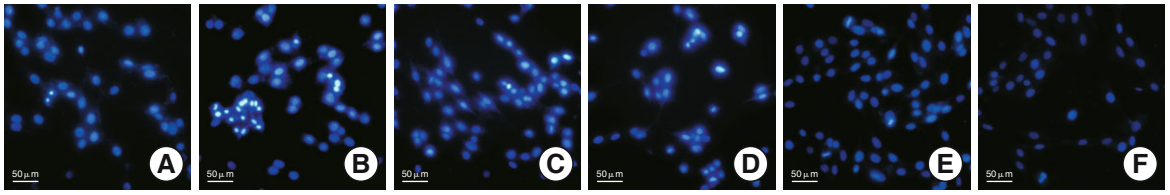

Figure 3. Effect of Sen on Morphological Changes in Nuclei of PC12 Cells

Notes: A: Cellular nucleis were homogeneously stained with Hoechst 33258 in control; B: Cells in the H/R group showed condensed nuclei and fragmental chromatin with bright blue fluorescence, and some cells of which showed pyknosis and karyorrhexis; C: H/R+Sen (15 μmol/L) group; D: H/R+Sen (30 μmol/L) group; E: H/R+Sen (60 μmol/L) group; F: Sen (60 μmol/L) group. Correspongding to the gradual increase of concentration of Sen treatment, the degree of pyknosis and karyorrhexis decreased gradually (×400, Bar=50 μmol/L).

staining. The apoptotic rate was significantly increased after H/R injury (29.53%±7.25% *vs*. 3.83%± 1.04%) compared with control group. When PC12 cells were treated with Sen (15, 30, 60 μmol/L), the percentages of apoptotic cells were decreased to 6.83%±1.65%, 4.67%±0.81%, 4.16%±1.19%, respectively as respected (Figure 4 and Table 1).

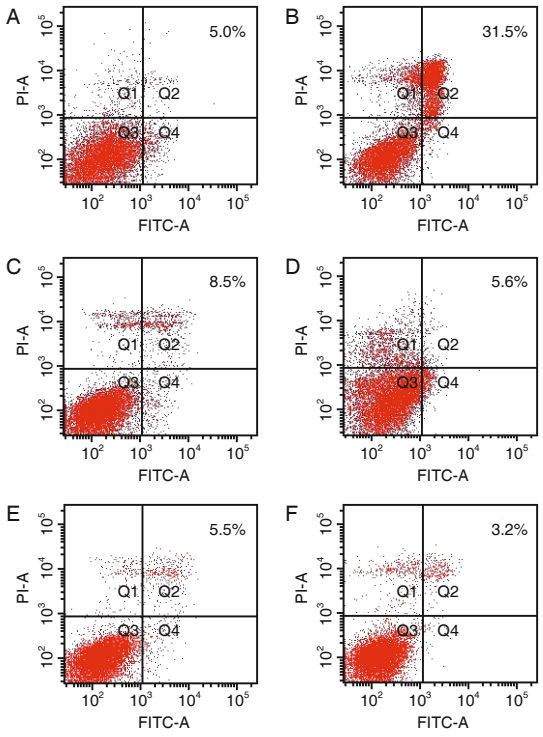

Figure 4. Effect of Sen against H/R-induced Apoptosis of PC12 Cells

Notes: A: Control group; B: H/R group; C: H/R+Sen (15 μmol/L) group; D: H/R+Sen (30 μmol/L) group; E: H/R+Sen (60 μmol/L) group; F: Sen (60 μmol/L) group

Sen Prevents the Loss of △Ψm

To explore the anti-apoptotic mechanism of Sen, the △Ψm with JC-1 staining, a cationic dye which exhibits a potential dependent accumulation in mitochondria was measured. Red fluorescence was exhibited as dominating fluorescence, an indicator of normality of △Ψm, in JC-1-stained cells in both control group and Sen (60 μmol/L) group (Figure 5). Green fluorescence was shown as dominating fluorescence, an indicator of dissipation of △Ψm, in JC-1-stained cells in the H/R group. With the concentration increasing in Sen, △Ψm rose gradually and JC-1-stained cells showed enhancing red fluorescent intensity compared with the H/R group (Figure 5 and Table 2). The △Ψm was next analyzed quantitatively by a flow cytometer. The results were consistent with our microscope data (Table 3).

Table 1. Effect of Sen against Apoptosis Rate of PC12 Cells Exposed to H/R Injury (*n*=3 in Each Grup, $\bar{x}\pm s$)

Group	Apoptosis rate (%)
Control	3.83±1.04
H/R	29.53±7.25*
H/R+Sen (15 μmol/ L)	6.83±1.65△
H/R+Sen (30 μmol/ L)	4.67±0.81△
H/R+Sen (60 μmol/L)	4.17±1.19△
Sen (60 μmol/L)	3.33±0.32

Notes: *P<0.05, compared with the control group; △P<0.05, compared with the H/R group

Effect of Sen on the Activity of Caspase-3 in H/R-Injured PC12 Cells

Cleaved caspase-3 was detected by colorimetric protease assay kit. The results showed that the quantity of activated caspase-3 was elevated dramatically by H/R treatment, while this change induced by H/R injury in PC12 cells was attenuated by Sen-treatment (15, 30, 60 μmol/L). And Sen took effect in a concentration-dependant manner (Figure 6).

Effect of Sen on Intracellular ROS, $[Ca^{2+}]_i$ Level and the Activity of NADPH Oxidase

The levels of intracellular ROS and $[Ca^{2+}]_i$ along with the activity of NOX in PC12 cells were evaluated

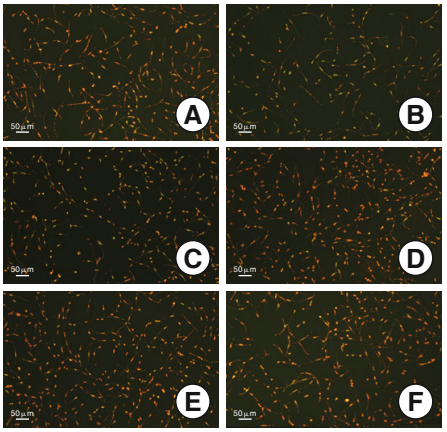

Figure 5. Sen Precluded the Loss of △Ψm in H/R-Injured PC12 Cells

Notes: A: Control group. JC-1-stained cells manifested red fluorescence predominantly, indicating aggregation of JC-1 in cells with intact △Ψm; B: H/R group. JC-1-stained cells in H/R group displayed green fluorescence, indicating existence of monomer of JC-1 in cells with △Ψm lost; C: H/R+Sen (15 μmol/L) group; D: H/R+Sen (30 μmol/L) group; E: H/R+Sen (60 μmol/L) group; F: Sen (60 μmol/L) group. As the concentration of Sen increased, intensity of red fluorescence in JC-1-stained cells augmented correspondingly (×100, Bar = 50 μmol/L).

Table 2. Effect of Sen on △Ψm of PC12 Cells Exposed to H/R Injury (Fluorescene Intensity by Microsope, *n*=3 in Each Group, $\bar{x} \pm s$)

Group	Ratio (Red/Green)
Control	0.31 ± 0.02
H/R	0.14 ± 0.01*
H/R+Sen (15 μmol/L)	0.16 ± 0.01
H/R+Sen (30 μmol/L)	0.22 ± 0.02△
H/R+Sen (60 μmol/L)	0.32 ± 0.03△
Sen (60 μmol/L)	0.34 ± 0.03

Notes: *P<0.05, compared with the control group; △P<0.05, compared with the H/R group

Table 3. Effect of Sen on △Ψm PC12 Cells Exposed to H/R Injury (Fluorescent Indensity by Flow Cytometry, *n*=3 in Each Group, $\bar{x} \pm s$)

Group	Ratio (Red/Green)
Control	2.27 ± 0.25
H/R	0.48 ± 0.11*
H/R+Sen (15 μmol/L)	0.54 ± 0.13
H/R+Sen (30 μmol/L)	2.19 ± 0.08△
H/R+Sen (60 μmol/L)	2.23 ± 0.11△
Sen (60 μmol/L)	2.27 ± 0.21

Notes: *P<0.05 compared with the control group; △P<0.05, compated with the H/R group

after H/R injury with or without Sen. As shown in Figure 7, the level of intracellular ROS and $[Ca^{2+}]_i$ as well as the activity of NOX in PC12 cell were markedly increased by H/R injury. However, such augments

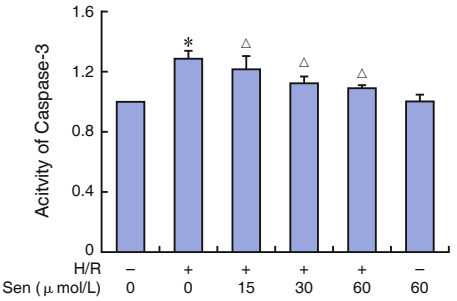

Figure 6. Effect of Sen on the Activity of Caspase-3 in H/R-Injured PC12 Cells

Notes: Values are $\bar{x} \pm s$ from three independent experiments. *P<0.05, compared with the control group; △P<0.05, compared with the H/R group

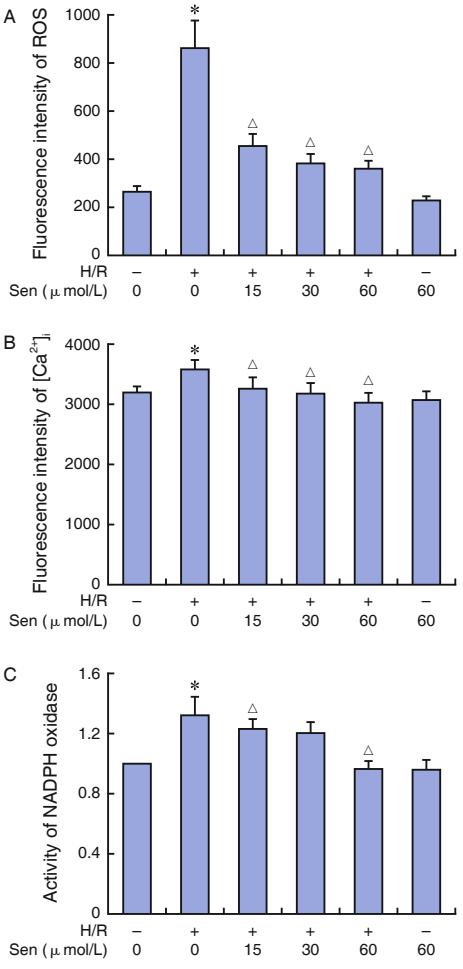

Figure 7. Effect of Sen on Intracellular ROS and $[Ca^{2+}]_i$ Level as Well as the Activity of NADPH Oxidase in H/R-Injured PC12 Cells

Notes: Values are $\bar{x} \pm s$ from three independent experiments. *P<0.05, compared with the control group; △P<0.05, compared with the H/R group

were inhibited by Sen.

DISCUSSION

H/R mimics ischemia/reperfusion condition

and provides a convenient model to investigate pharmacological effect of drugs for neuroprotective potency. PC12 cells, which derive from rat pheochromocytoma, are extensively used as a neuronal model system *in vitro*. More evidences suggest that apoptosis may attribute significantly to cell death in I/R or H/R injury.[19,20] The classic features of apoptosis, cell shrinkage, chromatin condensation, and formation of apoptotic bodies, were observed in our model of H/R injury as well. Our present studies clearly demonstrated that Sen could reduce the H/R-induced apoptosis rate. The result of Hoechst 33258 staining revealed that the number of cells with pyknosis and karyorrhexis was significantly reduced after treatments with Sen of different concentrations.

Mitochondria are well known as the principal coordinators in apoptotic process to control the intrinsic apoptotic pathway. This intrinsic apoptotic pathway can be triggered by a range of pathological conditions including H/R-induced injury.[13] The loss of $\triangle\Psi$m, the parameter of mitochondrial, has been employed to be hallmarks of mitochondria-dependent apoptosis.[14] The $\triangle\Psi$m measured semi-quantitatively by a fluorescent microscope suggested that Sen could maintain the red fluorescent intensity exposed to H/R injury in a dose-dependent manner. The $\triangle\Psi$m which was determined quantitatively by flow cytometer, showed a higher ratio of red/green fluorescent intensity in the H/R+Sen group than in the H/R group.

In addition, opening of permeability transition pore, loss of $\triangle\Psi$m and activation of caspase-9 and caspase-3 are involved in the mitochondria-dependent apoptotic pathway. Caspase-3, acting as a final executor, can activate DNA fragmentation factor, which in turn activate endonucleases to cleave nuclear DNA, and ultimately leads to cell apoptosis. It was noted in this study that H/R exposure increased the activation of casepase-3, and Sen effectively attenuated this change in PC12 cells exposed to H/R in a dose-dependent manner.

It has been well verified that significant amounts of oxygen free radicals, especially ROS, are generated during cerebral I/R, and oxidative stress plays a vital role in neuronal cell apoptosis mediated by I/R injury.[15] Meanwhile, mitochondria are deemed as a sensor of oxidative stress. Increased level of ROS has been demonstrated to induce depolarization of the mitochondrial membrane, which eventually increases the level of other pro-apoptotic molecules in cells.[21] Moreover, mitochondria have been previously identified as a primary source of ROS following I/R.[22] Thus the intracellular level of ROS was detected. The result indicated that the level of intracellular ROS could be markedly elevated by H/R injury, while this process was effectively mitigated by Sen. It is well known that overproduction of ROS causes numerous deleterious effects, such as a weakened cell antioxidant defense and an increased membrane permeability. The level of intracellular Ca^{2+} increased as a consequence. Wang, et al[23] and Son, et al[24] addressed that the elevation of cytosol Ca^{2+} could induce mitochondrial membrane permeability transition which facilitated the loss of $\triangle\Psi$m and eventually stimulated ROS production. ROS, with its positive stimulation of mitochondrial Ca^{2+} signals, may lead to further augment of cytosol Ca^{2+}. This positive feed-back loop of calcium and ROS occurs in the programmed cell death of H/R-induced injury. It was manifested in our present study that the level of intracellular Ca^{2+} increased significantly in the H/R group, while Sen treatments dramatically hindered this change.

It is well demonstrated in previous studies that inhibition of ROS production or scavenging of ROS offers protection against injury during I/R. Some of these studies have used chemical or enzymatic scavengers of ROS,[25] whereas others aimed at inhibiting the source(s) responsible for the production of ROS.[26,27] Recent evidence shows that NADPH oxidase contributes significantly to ROS generation following I/R as well.[16,28] It was observed in previous studies that genetic deletion of NADPH oxidase confers protection against ischemia-induced ROS production in the lung in gp91phox-/- mice.[29] NADPH oxidases of the NOX family are related to the production of ROS in various cells.[29-31] Sen was supposed to realize its neuroprotection via suppressing the activity of NADPH oxidase, thereby decreasing intracellular ROS production. The present results are consistent with our presumption: Sen significantly reduced the activity of NADPH oxidase in PC12 cells exposed to H/R injury. It suggests that the protective mechanism of Sen may be relevant to the inhibition of the production of ROS.

In sum, the massive production of ROS during H/R is caused by the activation of NADPH oxidase. Large amounts of ROS lead to diversified detrimental effects including the loss of $\triangle\Psi m$, intracellular Ca^{2+} overload and the activation of caspase-3, thus opened the pathways to cell apoptosis. It was demonstrated in our study that Sen could exert neuroprotection against H/R-induced injury via suppression key molecules mentioned above.

Conflict of Interest

The authors declare that they have no conflicts of interests. All authors disclose that there are no financial and personal relationships with other people or organizations.

Author Contributions

Qi RB participated in the design of the study, selection of journals and assessment of the manuscript. Zhu XQ carried out experiments and wrote the manuscript. Li XM and Ji XL assisted to the experiments and analyzed the dates. Wang YP, Fu YM, and Lu DX participated in the date analysis. All authors approved the final manuscript.

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