Research Article
Polydatin Protects Bone Marrow Stem Cells against Oxidative Injury: Involvement of Nrf 2/ARE Pathways

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Polydatin (Figure 2(a)), isolated from the roots of Polygonum cuspidatum, is widely used in traditional Chinese remedies [11–14]. Polydatin has been shown to protect heart function, prevent the development of diabetic renal fibrosis, and ameliorate Alzheimer's disease due to its multiple pharmacological actions, such as antioxidation, anti-inflammation, immunoregulation, antitumor, and neuroprotection [15–18]. However, the protective activity of polydatin on transplanted BMSCs after SCI is unknown.

In this study, we demonstrated for the first time that polydatin might protect BMSCs against \( H_2O_2 \)-induced apoptosis due to enhancing the resistance of BMSCs against oxidative injury and activate the nuclear factor E2-related factor 2 (Nrf 2)/antioxidant response element (ARE) pathway, which has been reported to have key roles in regulating endogenous antioxidants and phase II detoxification enzymes, suggesting that polydatin could be a promising approach to increase the cell survival in cell replacement therapy for SCI.

1. Introduction

Among central nervous system (CNS) disorders, spinal cord injury (SCI) is the most devastating and traumatic [1, 2]. 40 cases per million individuals are diagnosed as SCI [3]. Bone marrow-derived mesenchymal stem cells (BMSCs), which possess immunosuppressive properties and the capacity for unlimited self-amplification and for terminal differentiation [4, 5], play a privileged role in ameliorating neuronal damage in CNS disease models including SCI [6]. Cellular replacement with MSCs in different SCI animal models has showed functional recovery [7, 8]. However, attempts to transplant BMSCs into animal and human subjects are hampered mainly due to the poor survival of BMSCs [9]. After being transplanted, BMSCs are facing a complicated environment with risk factors that may lead to cell death including oxidative stress [4, 9, 10]. The increased reactive oxygen species (ROS) resulting in sustained oxidative stress in damaged spinal cord is one of the key factors that challenged the survival of donor BMSCs. BMSCs may unavoidably result in apoptosis under oxidative circumstance. Therefore, drugs with antioxidative effects and antiapoptosis may be crucial for the successful transplantation of BMSCs in SCI [10].

Polydatin (Figure 2(a)), isolated from the roots of Polygonum cuspidatum, is widely used in traditional Chinese remedies [11–14]. Polydatin has been shown to protect heart function, prevent the development of diabetic renal fibrosis, and ameliorate Alzheimer's disease due to its multiple pharmacological actions, such as antioxidation, anti-inflammation, immunoregulation, antitumor, and neuroprotection [15–18]. However, the protective activity of polydatin on transplanted BMSCs after SCI is unknown.

In this study, we demonstrated for the first time that polydatin might protect BMSCs against \( H_2O_2 \)-induced apoptosis due to enhancing the resistance of BMSCs against oxidative injury and activate the nuclear factor E2-related factor 2 (Nrf 2)/antioxidant response element (ARE) pathway, which has been reported to have key roles in regulating endogenous antioxidants and phase II detoxification enzymes, suggesting that polydatin could be a promising approach to increase the cell survival in cell replacement therapy for SCI.

2. Materials and Methods

2.1. Materials. Male Sprague-Dawley (SD) rats (100 ± 20 g) were supplied by the Center of Experimental Animals,
Guangzhou University of Chinese Medicine (Guangzhou, China, Certificate number 00100561). All procedures were performed according to animal guidelines of Guangzhou University of Chinese Medicine. Polydatin was purchased in Aladdin (Shanghai, China). Trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoched 33258, and dichlorofluorescein diacetate (H₂DCF-DA) were purchased from Sigma-Aldrich (MO, USA). Low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (NY, USA). Hydrogen peroxide, lactate dehydrogenase (LDH), Annexin V FITC/PI, Cell-Light 5-ethyl-2'-deoxyuridine (EdU) Apollo594 in vitro Image kit, and glutathione (GSH) assay kits were purchased from Keygen (Nanjing, China). Brusatol was bought from Chengdu PureChem-Standard Co., Ltd. (Chengdu, China). Polydatin (Aladdin) was dissolved in DMSO before dilution with the culture medium. The final concentration of DMSO was 0.1%.

2.2. Cell Culture and Treatment. Culture of rat BMSCs was performed as previously described [19]. Briefly, all bone marrow was flushed out with a 10 mL syringe using LG-DMEM supplemented with 10% FBS. The whole marrow washouts were collected, centrifuged, and plated into a culture flask in 37°C under 5% CO₂. All cells used in the assay were of passages 3–5. The phenotypic properties of BMSCs were identified by flow cytometry as previously reported [20]. Cells were pretreated with polydatin for 2 h and then treated with H₂O₂ (600 μM) for 24 h.

2.3. Cell Viability Assay. Cell viability was measured by MTT assay. Cells were plated on 96-well plates at a density of 1 x 10⁴ for 24 h. After incubation with H₂O₂ for 24 h, 10 μL MTT (5 mg/mL) was then added to each well and the mixture was incubated for 3 h at 37°C. MTT reagent was then replaced with DMSO (100 μL per well) to dissolve formazan crystals. After the mixture was shaken at 37°C for 15 min, absorbance was determined at 570 nm using a microplate reader. Results were expressed as the percentage of MTT reduction and the absorbance of control cells was set as 100%.

2.4. LDH Release Assay. The cytotoxicity was measured by LDH release assay. LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membrane and released from cells with damaged membranes. After the indicated treatment of BMSCs, the medium was collected and assayed for LDH activity as previously reported [21]. Briefly, the release of LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into red-colored formazan, which is correlated with LDH activity. The formazan was measured with a microplate reader at 450 nm. Results were expressed as the percentage of LDH release and the absorbance of control cells was set as 100%.

2.5. Hoched 33258 Assay. To detect morphological evidence of apoptosis, cell nuclei were visualized by DNA staining with the fluorescent dye Hoched 33258. After treatment, BMSCs were stained with Hoched 33258 (1 μg/mL) for 15 min in the dark. Results were tested by visual observation of nuclear morphology through fluorescence microscopy (Olympus, Japan) equipped with a UV filter.

2.6. Annexin V-FITC Assay. The apoptotic ratios of cells were determined with the Annexin V-FITC apoptosis detection kit. Briefly, BMSCs were collected and washed twice with cold PBS buffer, resuspended in 500 μL of binding buffer, incubated with 5 μL of Annexin V-FITC, conjugated to FITC and 5 μL PI for 15 min at room temperature, and analyzed by flow cytometry I (BD Biosciences). Cells treated with DMSO were used as the negative control.

2.7. Measurement of ROS. Intracellular ROS formation was measured using H₂DCF-DA as reported [22]. Briefly, after treatment, cells were washed with warm PBS three times and then stained with 10 μM H₂DCF-DA in serum-free medium for 30 min at 37°C in the dark. DCF fluorescence was analyzed by visual observation of cell morphology through fluorescence microscopy equipped with a UV filter.

2.8. Detection of Intracellular GSH. Intracellular GSH concentration was tested by a GSH assay kit. By reacting with dithiobis-nitrobenzoic acid, reduced GSH could form a yellow compound, which is quantifiable at 405 nm and is related to the concentration of the reduced GSH. In brief, whole-cell lysate was prepared according to manufacturer’s instructions. The basal contents of GSH in control cells were taken as 100%.

2.9. Cell Proliferation. The proliferation of BMSCs was tested with EdU assay. BMSCs were planted into 6-well plate, and then cells were allowed to adhere for 24 h. After the treatment, BMSCs were incubated with EdU for 4 h before fluorescent detection. Cells were fixed with 2% paraformaldehyde for 15 min and stained with EdU kit according to the manufacturer’s instructions. Finally, cells were placed under a laser-scanning confocal microscope (LSM710, Carl Zeiss, Germany) for image acquisition.

2.10. Western Blot Analysis. Western blotting analysis was performed as previously described [22]. In brief, cellular protein was collected and lysed in lysis buffer. The protein concentration was measured using the BCA assay (Keygen, Nanjing, China). Equal amounts of total protein were separated on SDS-PAGE gel and transferred onto the PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies Bcl-2, Bax, Nef2, and NQO-1 (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C, followed by sequential incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h. The bands were visualized by an enhanced chemiluminescence detection kit (ECL, Amersham Arlington Heights, IL, USA) and exposed to gel imaging system. The intensities of bands were performed using Quantity One Software (Bio-Rad, Hercules, CA).
2.11. Statistical Analysis. The data were presented as mean ± S.E.M. Statistical analyses between two groups were performed by unpaired Student’s t-test. Differences among groups were tested by one-way analysis of variance (ANOVA). A probability value of \( p < 0.05 \) was accepted to be statistically significant.

3. Results

3.1. Characterization of BMSCs. BMSCs were isolated from rat bone marrow, expanded in primary culture and passaged for three times. At initial phase, BMSCs of growth contained attached spindle-shaped cells with colonies and floating cells (Figure 1(a)), reaching confluence at day 7 (Figure 1(b)). The floating cells were completely abolished at passage 3 (Figures 1(c) and 1(d)).

3.2. Effects of Polydatin on BMSCs Exposed to \( \text{H}_2\text{O}_2 \). The viability of BMSCs treated with \( \text{H}_2\text{O}_2 \), ranging from 400 to 800 μM for 24 h, decreased dose-dependently. 600 μM \( \text{H}_2\text{O}_2 \) caused approximately cell death by 50% (Figure 2(b)) and the concentration was chosen for the following experiments. To investigate the effects of polydatin on \( \text{H}_2\text{O}_2 \)-induced cell death, MTT and LDH assays were applied. The results showed that polydatin significantly increased cell viability (Figures 2(c) and 2(d)) and decreased cell death (Figure 2(e)).

3.3. Polydatin Reduced \( \text{H}_2\text{O}_2 \)-Induced Apoptosis-Like Cell Death. Hochest 33258 staining and Annexin V-propidium iodide (PI) staining assay were used to observe whether \( \text{H}_2\text{O}_2 \) induced apoptotic death. Our results showed that \( \text{H}_2\text{O}_2 \) induced nuclear condensation (Figure 3(a)), which was blocked by polydatin. The total apoptotic rate (total rate of the cells that are Annexin V positive and PI positive) of \( \text{H}_2\text{O}_2 \) group (10.95% ± 1.25) was significantly increased compared with control group (4.45% ± 0.15), and polydatin effectively reduced the apoptotic rate (5.15% ± 0.75) (Figures 3(b) and 3(c)). Furthermore, after treatment with \( \text{H}_2\text{O}_2 \), upregulation of proapoptotic protein Bax and cleaved caspase-3 and downregulation of antiapoptotic protein Bcl-2 were observed in BMSCs, which were reversed by polydatin pretreatment.
3.4. Polydatin Decreased the Intracellular ROS Formation. To further disclosure the protective mechanism of polydatin, we detected its effects on the formation of intracellular ROS by H2DCF-DA staining, a ROS probe, and the endogenous antioxidant glutathione (GSH) using a GSH assay kit. As shown in Figures 4(a) and 4(b), compared with the control group, H2O2-treated group cause significant increase of ROS, which was attenuated by polydatin. Moreover, polydatin also improve the intracellular GSH which was depleted by H2O2 (Figure 4(c)).

3.5. Effects of Polydatin on the Cell Cycle of BMSCs. It is reported that polydatin, the natural precursor of resveratrol, inhibits proliferation of tumor cells caused by the cell cycle arrest [23, 24]. Thus, the survival effect of polydatin indicated in the study might simply be a switch of MSCs into
Figure 3: Polydatin attenuated H$_2$O$_2$-induced apoptosis in BMSCs. (a) Hoechst 33258 staining was applied to detect the nuclear condensation of BMSCs, pretreated with polydatin in presence of H$_2$O$_2$. Fluorescence images (A–D) were observed by fluorescence microscope. (A)–(D) represented CT, polydatin, H$_2$O$_2$, and H$_2$O$_2$ + polydatin group, respectively. (b) BMSCs were pretreated with 30 μM polydatin for 2 h and followed by exposing to H$_2$O$_2$ (600 μM) for 12 h. The induction of apoptosis was determined using Annexin V-FITC/PI staining. (c) Quantitative analysis of apoptotic cells in Figure 3(b). (d) The expression of Bcl-2, cleaved caspase-3, and Bax of BMSCs exposed to H$_2$O$_2$ with or without polydatin. Data are presented as means ± S.D. * $p < 0.05$ versus control group; # $p < 0.05$ versus H$_2$O$_2$-treated group.

quiescence. To examine whether the protective effects are related to polydatin cell cycle arrest activities, EdU assay was applied. Our results showed that H$_2$O$_2$ significantly reduced the proliferation rate of BMSCs compared with control group; polydatin at 30 μM did not cause proliferation inhibition on BMSCs, which suggests that polydatin may not lead to cell cycle arrest on BMSCs at the concentration (Figure 5).

3.6. Polydatin Prevented BMSCs from H$_2$O$_2$-Induced Apoptosis through Nrf 2/ARE Pathway. Polydatin has been reported to quench ROS overproduction by activating Nrf 2/ARE pathway, which has been reported to have key roles in regulating a battery of endogenous antioxidants and phase II detoxification enzymes, including NAD(P)H quinone oxidoreductase-1 (NQO-1) [25]. To explore whether Nrf 2/ARE pathway was involved in the protection of polydatin against oxidative injury, Western blotting was applied. As shown in Figures 6(a)–6(c), H$_2$O$_2$ significantly decreased the protein levels of p-Nrf 2 and NQO-1 protein, which was partly reversed by polydatin.
To further confirm the involvement of Nrf2/ARE pathway in the protective effects of polydatin, brusatol, a unique inhibitor of the Nrf2 pathway, which selectively reduces the protein level of Nrf2 through enhanced degradation and ubiquitination of Nrf2, was applied [26, 27]. As shown in Figure 6(d), brusatol at 100 nM significantly reduced phosphorylation of Nrf2 and did not cause cell death in BMSCs. Therefore, the concentration was chosen for the next experiment. Our results showed that polydatin attenuated cell viability decrease caused by H$_2$O$_2$, which was reversed by brusatol (Figure 6(h)). Moreover, brusatol also blocked the ROS scavenging activities of polydatin (Figures 6(f) and 6(i)).

4. Discussion

To the best of our knowledge, this is the first report about the effects of polydatin on the oxidative injury induced by H$_2$O$_2$ in BMSCs. We observed that polydatin dramatically attenuated H$_2$O$_2$-induced ROS generation, GSH depletion, LDH release, and subsequent cell death. Further studies showed that polydatin also enhanced phosphorylation of Nrf2 and upregulation of NQO-1 which was downregulated by H$_2$O$_2$, suggesting that polydatin might protect BMSCs against H$_2$O$_2$ partly via Nrf2/ARE pathway.

BMSCs, which are capable of self-renewal and differentiation into a variety of mesodermal cell lineages, including osteocytes, chondrocytes, myoblasts, and adipocytes [28, 29] are considered as an ideal source of cells for cell replacement therapy. BMSCs transplantation has shown great promises for treating vast CNS disorders, including SCI. However, poor viability of transplanted BMSCs in injured spinal cord has limited the therapeutic efficiency. Oxidative stress is one of the key mechanisms underlying the pathogenesis of CNS disorders including SCI. Sustained oxidative stress could reduce the survival of donor BMSCs, causing limited reparative capacity of BMSCs. Therefore, it is rational to improve the poor oxidative environment and protect the BMSCs against oxidative stress for the successful transplantation of BMSCs in SCI.

Polydatin, an active stilbene compound isolated from the roots of *Polygonum cuspidatum* Sieb. and Zucc., has been shown to prevent the development of diabetic renal fibrosis, ameliorate Alzheimer’s disease, and protect ischemia/reperfusion damage in heart and diabetic nephropathy. It has also been reported to have antiapoptosis and antioxidation activities in many cellular systems. However, protective effects of polydatin on BMSCs are unknown. We used H$_2$O$_2$ to induce oxidative injury on BMSCs, imitating the
poor microenvironment of the spinal cord after SCI. Our results showed that H$_2$O$_2$ reduced cell viability of BMSCs dose-dependently and caused a robust ROS generation and GSH depletion as previously reported [10]. Polydatin, at a concentration of 30 $\mu$M, effectively suppressed H$_2$O$_2$-induced cell death, scavenged the ROS, and reversed the depletion of GSH, indicating that polydatin exerts beneficial effects on BMSCs as well.

Bcl-2 and Bax are two members of the Bcl-2 family, which are crucial regulatory factors in apoptosis. Bcl-2, the antiapoptotic protein, inhibits apoptosis by preventing cytochrome c release into the cytoplasm [22], while Bax, the proapoptotic protein, promotes apoptosis by inducing mitochondrial membrane depolarization. The Bcl-2 family maintains mitochondrial stabilization by mediating the Bcl-2/Bax balance [30]. Caspase-3 is a pivotal executioner caspase, which triggers the cleavage of a number of proteins and ultimately leads to DNA fragmentation, and has long been considered as a key protease involved in cell apoptosis [31]. In the present study, we examined the underlying mechanism of the protection of polydatin against H$_2$O$_2$-induced apoptosis by detecting the expression of apoptosis-related proteins using Western blot. We observed the upregulation of Bax and cleaved caspase-3 and downregulation of Bcl-2 following treatment of H$_2$O$_2$, which were overtly reversed by polydatin, suggesting its antiapoptotic effects.

It is well established that polydatin (also named piceid) and resveratrol inhibit proliferation of tumor cells caused by the cell cycle arrest [23, 24]. Thus, the survival effect of polydatin indicated in the study might simply be a switch of MSCs into quiescence. To examine whether the protective effects are related to polydatin cell cycle arrest activities, we detected the proliferation rate of BMSCs pretreated with polydatin in the presence or absence of H$_2$O$_2$ using EdU assay. The results showed that polydatin at 30 $\mu$M did not cause proliferation inhibition on BMSCs, which suggests that polydatin may not lead to cell cycle arrest on BMSCs at the concentration. According to Su et al., polydatin induced the cell cycle arrest in the S phase at 300 $\mu$M on MDA-MB-231 cells but not MCF-7 cells and HepG2 cells, suggesting that polydatin only cause proliferation inhibition in certain cell lines at proper concentrations [23]. According to Su et al., polydatin protected MDA-MB-231 cells against H$_2$O$_2$ toxicity at 50 $\mu$M, a concentration which did not cause cell cycle arrest, indicating that the protective effects of polydatin were independent from its effects on cell cycle arrest. Therefore, the protective effects of polydatin reported in our paper may be just related to its antioxidative activities.

Nrf 2, a basic leucine zipper transcription factor, is reported to drive transcription of all kinds of genes involved in combating products of oxygen radicals and oxidation such as protein and DNA adducts from carbonyls or malondialdehyde [25, 32]. Under normal conditions, Nrf 2 binds to Kelch-like ECH associated protein-1 (Keap1) [33]. When oxidative stress occurs, Nrf 2 is released from Keap1, is translocated to the nucleus, is bound with ARE sequences, and results in transcriptional activation of antioxidant genes including NAD(P)H quinone oxidoreductase-1 (NQO-1) [34]. Huang et al. have reported that polydatin activated Nrf 2/ARE pathway in glomerular mesangial cells [16]. Herein, we found that H$_2$O$_2$ downregulated NQO-1 and the phosphorylation of Nrf 2 which was partly reversed by polydatin. To further confirm the involvement of Nrf 2/ARE pathway in the protection of polydatin, brusatol was applied. Previous studies reported...
Figure 6: Continued.
brusatol as a unique inhibitor of the Nrf 2 pathway, which selectively downregulates the protein level of Nrf 2 via increasing ubiquitination and degradation of Nrf 2 [27]. Herein, we proved that coincubation with polydatin and brusatol reversed the protective and the ROS scavenging effects of polydatin, suggesting that Nrf 2/ARE pathway was involved in the protection and antioxidation of polydatin against H₂O₂-induced cell death.

5. Conclusion

Taken together, our results indicate that polydatin exerts strikingly protective effects against H₂O₂-induced cytotoxicity in BMSCs through activating the Nrf 2/ARE pathway, suggesting that polydatin could be a promising approach to increase the cell survival in cell replacement therapy for SCI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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