Research Article

Preparation of Ginsenoside Rg3 and Protection against H₂O₂-Induced Oxidative Stress in Human Neuroblastoma SK-N-SH Cells

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The aim of this study is to evaluate the protection of ginsenoside Rg3 against oxidative stress in human neuroblastoma SK-N-SH cells. 20(R)-ginsenoside Rg3 (20(R)-Rg3) and 20(S)-ginsenoside Rg3 (20(S)-Rg3) were prepared by the method of chemical degradation and column chromatography, and the structure of the two compounds was characterized by ¹H-NMR and ¹³C-NMR spectroscopy. MTT assay and LDH leakage assay were used to determine the cell viability and the oxidative stress cellular model was established by means of H₂O₂ (600 μM for 4 h). We also investigated the changes of intracellular MDA content, SOD activity, and ROS formation after the treatment of ginsenoside Rg3 for 20 h. The results indicated that both 20(R)-Rg3 and 20(S)-Rg3 had obvious protection against H₂O₂-induced oxidative stress in SK-N-SH cells. Moreover, 20(R)-Rg3 exhibited better antioxidant activity than 20(S)-Rg3 *in vitro*. These findings are expected to provide some implication for further research and application of ginsenoside Rg3 in neuroprotection.

1. Introduction

Panax ginseng is known to have various pharmacological activities [1]. Among several products of ginseng, ginsenosides are the main components and have been proved to possess many bioactivities, which mostly including antioxidation, neuroprotection, and anticancer [2].

Ginsenoside Rg3, a saponin extracted from ginseng, had been recently reported to protect the neuronal cells and animals with neurological injuries [3–6]. Ginsenoside Rg3 exists as stereoisomer of 20(R)- or 20(S)-form (Figure 1) and with different bioactivity [7, 8]. The two compounds can be biotransformed by human intestinal bacteria [9, 10]. In recent years, researchers have found the protection of different Rg3 isomer on nervous system. Tian et al. have investigated the neuroprotective effects of 20(S)-Rg3 on focal cerebral ischemia in rats and found that 20(S)-Rg3 could inhibit the opening of mitochondrial permeability transition pore by free radical scavenging action in the rats brain [11, 12].

He et al. have investigated the neuroprotective effect of 20(R)-Rg3 against transient focal cerebral ischemia in male Sprague-Dawley rats and finally indicated its neuroprotective effect may be involved in the downregulation of calpain I and caspase-3 [13]. However, rare reports have been found on the comparison study of 20(R)-Rg3 and 20(S)-Rg3, especially in the study of antioxidant and neural protection.

In the present study, we prepared the stereoisomer of 20(R)-Rg3 and 20(S)-Rg3 by chemical conversion method and characterized the structure by ¹H-NMR and ¹³C-NMR spectroscopy. Human neuroblastoma SK-N-SH cells are often used as *in vitro* models of neuronal function and differentiation [14, 15]. Here, we established the oxidative stress model by using SK-N-SH cells exposed to H₂O₂, and the protection of ginsenoside Rg3 against H₂O₂-induced oxidative stress was evaluated by the measurement of lactate dehydrogenase (LDH) release, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and intracellular reactive oxygen species (ROS) level. Some implication is

FIGURE 1: Structural formulas of 20(R)-Rg3 and 20(S)-Rg3.

expected to be provided for the further research and application of ginsenoside Rg3 in neuroprotection through this study.

2. Materials and Methods

2.1. Cells and Chemicals. Human neuroblastoma SK-N-SH cells were obtained from the cell bank of the Institute of Biochemistry and Cell Biology in Shanghai, China. Phosphate buffered saline (PBS), MEM medium, fetal bovine serum (FBS), penicillin-streptomycin solution, and Trypsin-EDTA solution were purchased from GIBCO Corporation (Beijing Representative Office, China). Ginseng stem-leave saponins (GSLs) were bought from Hongjiu Biological Technology Co., Ltd, in Jilin, China. Other chemical reagents were analytically pure and were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China.

2.2. Preparation of Ginsenoside Rg3. 20 g of GSLs was dissolved with 500 mL of acetic acid water (30:70) and extracted under reflux at 90°C for 1 h. The extraction mixture was then filtered. The residue was washed using ethanol and recrystallized from methanol water. Finally, 1.48 g target compound was obtained. The structure of the compound was confirmed by ¹H-NMR and ¹³C-NMR as 20(R)-ginsenoside Rg3 (20(R)-Rg3), 20(R)-protopanaxadiol-3-O-beta-D-glucopyranosyl(1 → 2)-beta-D-glucopyranoside.

20 g of GSLs was mixed with 1000 mL of glycerin and 100 g of sodium hydroxide and reacted at 210°C for 2 h. As the solution cooled, pH was adjusted to 7.4. Then the precipitation was concentrated to dryness in vacuum and 7.7 g of gray degradation was obtained. Then, the degradation underwent silica gel column chromatography and was eluted with ethyl acetate:methanol (8:2). The resulting eluate was combined into two fractions (Fr1 and Fr2) based on the silica gel TLC profiles of each tube. The Fr2 was then purified again with chloroform:methanol (7:3). The final compound was recrystallized with methanol and confirmed by ¹H-NMR and ¹³C-NMR as 20(S)-ginsenoside Rg3 (20(S)-Rg3), 20(S)-protopanaxadiol-3-O-beta-D-glucopyranosyl(1 → 2)-beta-D-glucopyranoside.

2.3. Cell Culture. Human neuroblastoma SK-N-SH cells were cultured in MEM medium that contained 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were seeded on Petri dishes (10 cm) and the medium was replaced every other day.

2.4. Cell Viability Assay. The cytotoxicity of ginsenoside Rg3 was detected by MTT assay. The oxidative damage of H₂O₂ to SK-N-SH cells was confirmed by measuring the release of the cytosolic enzyme LDH to the culture medium. And the protective effect of ginsenoside Rg3 against toxicity of H₂O₂ was also confirmed by both MTT assay and LDH assay.

2.4.1. MTT Assay. The cytotoxicity of ginsenoside Rg3 was determined by the MTT assay as described [16]. SK-N-SH cells were plated in triplicate at a density of 1 × 10⁴ cells/well in 96-well plates for 24 h. The culture media were aspirated and replaced with fresh culture media containing various concentrations of 20(R)-Rg3 or 20(S)-Rg3 (1.5~200 µg/mL) for 20 h. Then, 10 µL MTT solution (5 mg/mL) was added into each well for further 4 h at 37°C. And 150 µL of DMSO was added in order to dissolve the formazan crystals. The UV absorbance of the solubilized formazan crystals was measured by microplate reader (Spectra MR, Dynex) at 490 nm. Cell viability (%) was calculated.

2.4.2. LDH Assay. The leakage of LDH was measured by using a colorimetric LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, 1 × 10⁴ cells/well were cultured in 96 well plates overnight. Then, the cells were treated with various concentration of H₂O₂ (200, 400, 600, 800, 1000, and 1200 µM) for different time (2 h, 4 h, and 6 h). Then, 20 µL of cell medium was added into basic solution to measure extracellular LDH activity, which could catalyze the conversion of lactate to pyruvate and react with 2,4-dinitrophenylhydrazine to give the brownish red color [17]. The absorbance was measured at 450 nm by automatic microplate reader.

As for the protective effect of ginsenoside Rg3, SK-N-SH cells were preincubated with 20(R)-Rg3 or 20(S)-Rg3

(10, 20 $\mu\text{g/mL}$) for 24 h before H_2O_2 oxidative damage, and the detection of LDH released into the culture medium was completed with the same process.

2.5. Oxidative Stress Analysis. After pretreatment with 20(R)-Rg3 or 20(S)-Rg3 for 24 h and subsequent treatment with 600 μM H_2O_2 for 4 h, H_2O_2 -induced oxidative stress and the protection of ginsenoside Rg3 were analyzed by cytosolic superoxide dismutase (SOD) assay, extracellular malondialdehyde (MDA) assay, and intracellular reactive oxygen species (ROS) detection.

2.5.1. SOD and MDA Determination. After treatment with ginsenoside Rg3 and H_2O_2 , the culture supernatant was collected, and MDA contents were measured using a spectrometer at 586 nm according to the manufacturer of the assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Then, the cells were detached with 0.25% trypsin and centrifuged at 1200 rpm. SOD activities were analyzed according to the instruction of the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at 550 nm.

2.5.2. Detection of Intracellular ROS. ROS production in SK-N-SH cells was measured using the redox-sensitive fluorescent dye DCFH-DA (Jiamei Biotechnology Co., Ltd., Beijing, China). After treatment with ginsenoside Rg3 and H_2O_2 , cells were incubated with 25 μM DCFH-DA for 20 min. The cells were detached with 0.25% trypsin and centrifuged at 1200 rpm. Then, the cells were rinsed twice with phenol-red-free MEM containing 1% FBS, and fluorescence was detected on automatic microplate reader (488 nm excitation and 520 nm emission). The mean fluorescent signals for 10,000 cells were recorded and the relative intracellular ROS level (%) was calculated.

2.6. Statistical Analyses. Data were expressed as mean $\pm$ S.D., and differences were evaluated using unpaired Student's *t*-tests or ANOVA by SPSS 18.0 software. The level of statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1. Chemical Structure Identification. The structural identification of ginsenoside Rg3 was carried out by electrospray ionization mass spectrometry (ESI-MS), ^1H NMR, and ^{13}C NMR spectra as follows.

20(R)-Rg3: ^1H -NMR (400 Hz, $\text{C}_5\text{D}_5\text{N}$), δ : 0.83 (3H, S, CH_3 -29), 1.01 (3H, S, CH_3 -30), 0.96 (3H, S, CH_3 -19), 1.12 (3H, S, CH_3 -18), 1.30 (3H, S, CH_3 -28), 1.66 (3H, S, CH_3 -27), 1.70 (3H, S, CH_3 -26), 4.63 (1H, d, $J = 7.4$ Hz), 5.31 (1H, d, $J = 7.4$ Hz); ^{13}C -NMR (400 Hz, $\text{C}_5\text{D}_5\text{N}$), δ : 39.1 (C-1), 26.7 (C-2), 88.9 (C-3), 39.9 (C-4), 56.3 (C-5), 18.4 (C-6), 35.1 (C-7), 36.9 (C-8), 50.6 (C-9), 39.7 (C-10), 32.2 (C-11), 70.9 (C-12), 49.2 (C-13), 51.7 (C-14), 31.4 (C-15), 28.1 (C-16), 50.4 (C-17), 16.6 (C-18), 72.9 (C-19), 22.8 (C-20), 43.3 (C-21), 22.6 (C-22), 126.0 (C-23), 130.8 (C-24), 25.8 (C-25), 17.7 (C-26), 26.6 (C-27), 15.8 (C-28), 17.3 (C-29), 105.1 (C-30), 83.5 (C-31), 71.6

FIGURE 2: Cytotoxicity effects of ginsenoside Rg3 on SK-N-SH cells.

(C-32), 77.9 (C-33), 62.6 (C-34), 106.1 (C-35), 77.1 (C-36), 78.3 (C-37), 71.6 (C-38), 78.1 (C-39), 62.8 (C-40).

20(S)-Rg3: ^1H -NMR (400 Hz, $\text{C}_5\text{D}_5\text{N}$), δ : 0.80 (3H, S, CH_3 -29), 0.95 (3H, S, CH_3 -30), 0.96 (3H, S, CH_3 -19), 1.11 (3H, S, CH_3 -18), 1.30 (3H, S, CH_3 -28), 1.61 (3H, S, CH_3 -27), 1.65 (3H, S, CH_3 -26), 4.83 (1H, d, $J = 7.6$ Hz), 5.29 (1H, d, $J = 7.2$ Hz); ^{13}C -NMR (400 Hz, $\text{C}_5\text{D}_5\text{N}$), δ : 39.1 (C-1), 27.0 (C-2), 88.9 (C-3), 39.9 (C-4), 56.3 (C-5), 18.4 (C-6), 35.8 (C-7), 36.9 (C-8), 32.0 (C-9), 71.6 (C-10), 48.5 (C-11), 51.7 (C-12), 39.7 (C-13), 32.0 (C-14), 71.6 (C-15), 48.5 (C-16), 51.7 (C-17), 32.0 (C-18), 26.8 (C-19), 35.1 (C-20), 23.0 (C-21), 126.3 (C-22), 130.7 (C-23), 25.8 (C-24), 17.6 (C-25), 28.1 (C-26), 16.3 (C-27), 17.0 (C-28), 105.1 (C-29), 83.4 (C-30), 78.3 (C-31), 71.6 (C-32), 77.2 (C-33), 62.7 (C-34), 105.1 (C-35), 77.1 (C-36), 78.3 (C-37), 72.8 (C-38), 77.9 (C-39), 62.8 (C-40).

3.2. Cell Viability Assay. As showed in Figure 2, both 20(R)-Rg3 and 20(S)-Rg3 had no obvious cytotoxicity on SK-N-SH cells when the concentration was 1.5–200 $\mu\text{g/mL}$. Intracellular LDH assay has proved that H_2O_2 could significantly lead to cell damage along with the extension of the time and with the increase of the concentration (Figure 3). After incubated with H_2O_2 , SK-N-SH cells were also found with different changes in shape through inverted microscope observation. Some cells were swelled, shrunken, and unevenly distributed. The cellular edge became unclear and even fell off when the damage was serious.

3.3. Protection of Rg3 against H_2O_2 -Induced Oxidative Stress. The results from MTT assay and intracellular LDH analysis had suggested that H_2O_2 -induced oxidative stress could lead to significant cell death. And previous ginsenoside Rg3 treatment was able to increase the cell survival rate and vitality with a dose-dependent manner (shown in Figures 4 and 5). Furthermore, the protective activity of 20(R)-Rg3 was significantly better than that of 20(S)-Rg3 ($P < 0.05$).

FIGURE 3: Effect of H₂O₂ on leakage of LDH in SK-N-SH cells.

FIGURE 4: Protective effect of ginsenoside Rg3 on SK-N-SH cells by MTT assay. ^{##}*P* < 0.01 versus normal group, **P* < 0.05, and ^{**}*P* < 0.01 versus model group.

MDA produced in cytoplasm was determined as a common indicator of lipid peroxidation [18]. H₂O₂ (600 μM for 4 h) could significantly increase the production of MDA generated in SK-N-SH cells. However, 20(R)-Rg3 and 20(S)-Rg3 could effectively reduce the content of MDA as showed in Figure 6.

SOD is an effective defense enzyme that catalyses the dismutation of superoxide anions into hydrogen peroxide [19]. Our results in the present study showed that the antioxidative activity of intracellular SOD was significantly decreased in H₂O₂ toxic groups compared with the normal cells (*P* < 0.01), which implied increased oxidative damage to the cells. On the contrary, SOD levels were significantly elevated by incubation of 20(R)-Rg3 and 20(S)-Rg3. These results demonstrated that the antioxidant ability of ginsenoside Rg3 was very critical for its protection on SK-N-SH cells. In addition, the protective effects of 20(R)-Rg3 were remarkable and powerful than that of 20(S)-Rg3 at the concentration of 20 μg/mL (*P* < 0.05) (Figure 7).

FIGURE 5: Protective effect of ginsenoside Rg3 on SK-N-SH cells by extracellular LDH assay. ^{##}*P* < 0.01 versus normal group, **P* < 0.05, and ^{**}*P* < 0.01 versus model group.

FIGURE 6: Ginsenoside Rg3 inhibited the MDA production in SK-N-SH cells. ^{##}*P* < 0.01 versus normal group, **P* < 0.05, and ^{**}*P* < 0.01 versus model group.

Oxidative stress reflects an imbalance between the systemic manifestation of ROS and the ability to repair the resulting damage by the biological system or the intake of exogenous antioxidants [20]. Given oxidative stress, the intracellular ROS levels would sharply increase which had been confirmed by the H₂O₂ exposure in the present study (Figure 8). Ginsenoside Rg3 could obviously scavenge the ROS produced by H₂O₂. We investigated the intracellular ROS formation by fluorescent probe DCFH-DA. The results showed that compared with the H₂O₂ damaged group, 10 and 20 μg/mL of 20(R)-Rg3 could decrease the ROS formation by 44.1% and 64.7%, respectively, meanwhile 10 and 20 μg/mL of 20(S)-Rg3 could decrease the ROS level by 29.2% and 51.3%, respectively.

4. Conclusions

H₂O₂ exposure (600 μM for 4 h) to human neuroblastoma SK-N-SH cells could significantly cause oxidative stress by reducing the intracellular SOD activity and promoting the MDA production and lead to further damage to cells. Ginsenoside Rg3, no matter 20(R)-Rg3 or 20(S)-Rg3, possesses

FIGURE 7: Ginsenoside Rg3 increased the SOD activity in SK-N-SH cells. ## $P < 0.01$ versus normal group, * $P < 0.05$, and ** $P < 0.01$ versus model group.

FIGURE 8: Ginsenoside Rg3 reduced the intracellular ROS formation in SK-N-SH cells. ## $P < 0.01$ versus normal group and ** $P < 0.01$ versus model group.

powerful ability of scavenging free radicals produced by H_2O_2 . Thus, they exhibited the strong protective effects on SK-N-SH cells. In addition, we also found in the present study that 20(R)-Rg3 displayed better antioxidant activity than 20(S)-Rg3 *in vitro*. These findings will benefit further research and application of ginsenoside Rg3 in neuroprotection.

Conflict of Interests

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

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