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Protective Effect of Ginsenoside R0 on Anoxic and Oxidative Damage *In vitro*

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Abstract

To examine the neuroprotective effects of ginsenoside R0, we investigated the effects of ginsenoside R0 in PC12 cells under an anoxic or oxidative environment with Edaravone as a control. PC12 neuroendocrine cells were used as a model target. Anoxic damage or oxidative damage in PC12 cells were induced by adding sodium dithionite or hydrogen peroxide respectively in cultured medium. Survival ratios of different groups were detected by an AlamarBlue assay. At the same time, the apoptosis of PC12 cells were determined with flow cytometry. The putative neuroprotective effects of ginsenoside R0 is thought to be exerted through enhancing the activity of antioxidant enzymes Superoxide dismutases (SOD). The activity of SOD and the level of malondialdehyde (MDA) and intracellular reactive oxygen species (ROS), were measured to evaluate the protective and therapeutic effects of ginsenoside R0. Ginsenoside R0 treated cells had a higher SOD activity, lower MDA level and lower ROS, and their survival ratio was higher with a lower apoptosis rate. It is suggested that ginsenoside R0 has a protective effect in the cultured PC12 cells, and the protection efficiency is higher than Edaravone. The protective mechanisms of these two are different. The prevent ability of ginsenoside R0 is higher than its repair ability in neuroprotection *in vitro.*

Key Words: Ginsenoside R0, Edaravone, Anoxic damage, Oxidative damage, PC12 cell

INTRODUCTION

Ginseng, from where the ginsenosides are extracted, is the root of *Panac ginseng*, and has been used in Asia for over 2000 years as a tonic in traditional Chinese medicine. Ginseng saponins isolated from ginseng had been regarded as the principal constituents responsible for the biological activities. So far, more than 40 different ginsenosides had been identified (Keum *et al.*, 2000). Ginsenosides have been known to cause several possible physiological effects including stimulating central nervous system, increasing the initial learning performance and anti-fatigue activity, promoting the activity DNA, protein and lipid synthesis in animal bone marrow cells and so on. Moreover, ginsenosides have been demonstrated to improve cardiovascular and nerve function (Lü *et al.*, 2009) by facilitating cholinergic function, increasing synaptophysin level in the hippocampus, and protecting the cultured cerebral cortex neurons against excitotoxicity (Kim *et al.*, 1998; Mook-Jung *et al.*, 2001; Liao *et al.*, 2002). The beneficial effects of ginsenoside Rb1 were mediated through scavenging the free radicals (Lim *et al.*, 1997), improving energy metabolism, and preserving the structural integrity of the neurons (Jiang and Qian, 1995). As a member of ginsenosides, ginsenoside R0 was not studied as much as other ginsenosides (Zhang *et al.*, 2001), it was reported about its anti-inflammatory activity (Matsuda *et al.*, 1990), anti-hepatitic activity (Matsuda *et al.*, 1991) and cell proliferation enhancement (Yu *et al.*, 2005). However, whether it has an effect on cell protection from free radicals is unclear. Therefore, in this study, we investigated the protective effects of ginsenoside R0 under an anoxic or oxidative environment *in vitro*.

MATERIALS AND METHODS

Cell culture and grouping

A *Rattus norvegicus* pheochromocytoma cell line, PC12 (PC12, ATCC® Number: CRL-1721.1™) cells were cultured in 1640 medium (Hyclone, USA) supplemented with antibiotics and 10% fetal calf serum (Hyclone, Ca, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°.

To compare the preventive effect and therapeutic effect

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of ginsenoside R0 with the classical free radical scavenger Edaravone Injection (3-Methyl-1-phenyl-2-pyrazolin-5-one) as a control, cells were divided into 3 main groups: A. Prevention group, in which cells were pretreated with ginsenoside R0 or Edaravone; B. Therapy group, in which cells treated with ginsenoside R0 or Edaravone after the anoxic or oxidative damage; C. Control group, in which cells were only with damage treatments or only treated with the ginsenoside R0 or Edaravone.

Prevention treatments

When cells of prevention group were at 85% confluence of each well in 6-well plates, 20 μl ginsenoside R0 (dissolved in 1,640 medium; Nanjingzelang pharmaceuticals company, China) with a concentration of 2.5 mg/ml, 7.5 mg/ml and 12.5 mg/ml, and 20 μl Edaravone (Huinanchanglong biochemical pharmaceuticals company, China) with a concentration of 1.0 mg/ml, 1.25 mg/ml and 1.5 mg/ml, were added in the medium of each well respectively for 1 day culture. They were marked as Prevention-R0-low, Prevention-R0-mid, Prevention-R0-high, and Prevention-E respectively.

Damage treatments

Anoxic or oxidative damage were done to the cells of therapy group and the pretreated prevention group by adding sodium dithionite (30 mM) and hydrogen peroxide (0.3 mmol/L) respectively. Sodium dithionite treatment lasted for 2h, while hydrogen peroxide treatment lasted for 30 min. They were marked as Damage-S and Damage-H respectively.

Therapy treatments

After damage treatments, mediums containing sodium dithionite or hydrogen peroxide were renewed, 20 μl ginsenoside R0 (2.5 mg/ml, 7.5 mg/ml and 12.5 mg/ml) or 20 μl Edaravone (1.0 mg/ml, 1.25 mg/ml and 1.5 mg/ml) were added into the new medium for therapy. They were marked as Therapy-R0-low, Therapy-R0-mid, Therapy-R0-high, andTherapy-E respectively.

Control treatments

Cells only treated with ginsenoside R0 or Edaravone were marked as Negative control Negative-R0 and Negative-E respectively; and the ones only treated with sodium dithionite or hydrogen peroxide were marked as Positive control Positive-S and Positive-H respectively.

AlamarBlue Assay

Survival of cells was determined by using AlamarBlue Assay (Back *et al.*, 1999; Lü *et al.*, 2009; Yang and Balcarcel, 2004). 20 μl AlamarBlue reagent (BioSource, Nivelles, Belgium) was added to 180 μl medium of each well in 96-well plate, and then kept the cell at 37°C for 4 h. Absorbance was monitored at 570 nm and reference wavelength at 600 nm in a microplate reader.

Flow cytometry

After different treatments, PC12 cells were detached and centrifuged at 1,000 rpm for 5 min to collect them, then resuspended in PBS and counted. Flow cytometry was performed to determine the apoptosis percentage of PC12 cells in different groups (Morimoto *et al.*, 2000) with Annexin V-FITC apoptosis determine kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instruction, using Beckman Cell Lab Quanta SC flow cytometry (Beckman, USA) and its corresponding software. Briefly, cells were detached and washed with PBS twice. 195 μl Annexin V-FITC combining solution was added to resuspended the cells, then added 5 μl Annexin V-FITC and mixed gently. The cells were incubated at 20-25°C for 10 min (avoiding light), then centrifuged at 1,000×g for 5 min. These cells were then resuspended in 195 μl Annexin V-FITC combining solution. 5-10×10^4 cells and counted. Using this, the percentage of the apoptotic cells accumulation in D2 was calculated.

MDA and SOD assay

The culture medium was collected, then cells were washed with D-Hanks, scraped from the plates into 1 ml of icecold PBS (0.1 M, containing 0.05 mM of EDTA), and homogenized. The homogenate was centrifuged at 4,000×g for 30 min at 4°C. The resulting supernatant and collected medium were used for analysis. The MDA and SOD were measured using their commercial ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Briefly, as for MDA, 100 μl of the supernatant/medium was mixed with 1.5 ml of acetic acid (20%, v/v, pH 3.5), 1.5 ml of thiobarbituric acid (0.8%, w/v), and 200 μl of sodium dodecyl sulphate (8%, w/v). Each reaction mixture was heated for 60 min at 95°C and cooled to room temperature. 5 ml of n-Butanol was then added. After mixing and centrifugation at 3,000×g for 10 min, the organic layer was collected and the absorbance measured at 532 nm; as for SOD, 20 μl of supernatant/medium was added with 200 μl of water soluble tetrazolium working solution and 20 μl of enzyme working solution to a 96-well plate. After incubating the plate at 37°C for 20 min, the absorbance at 550 nm was read using a microplate reader.

Determination of intracellular ROS

Intracellular ROS levels were determined with Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, cells with different treatments were washed with PBS and incubated with DCFH-DA at 37°C for 30 min. DCF fluorescence distribution of these cells was detected by an FLx800 Fluorescence and Luminescence Reader (BioTek Instruments, Vermont, USA) at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

Statistical analysis

All experiments were performed in triplicate and the results were shown graphically as Mean ± SD. The statistical significance of differences between groups was calculated by Student's *t*-test. Probability values (p) of less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS software (version 11.0, SPSS Inc.).

RESULTS

The viability of the cells

The viability of each group of the PC12 cells was detected by AlamarBlue assay, and was presented as survival ratio. This survival ratio was significantly low in cells which were only treated with sodium dithionite (Positive-S group) or hydrogen peroxide (Positive-H group), compared to the cells

which only treated with ginsenoside R0 (Negative-R0) or Edaravone (Negative-E) ($p<0.05$), as shown in Fig. 1. The cells which had been treated with ginsenoside R0 before (Prevention-R0-Damage-S group) or after (Damage-S-Therapy-R0 group) the anoxic damage had a better survival ratio than that in Positive-S group ($p<0.05$, Fig. 1A), and the results were similar Positive-H group ($p<0.05$, Fig. 1C). The difference between Prevention-R0-Damage-S and Damage-S-Therapy- R0 groups was not significant ($p>0.05$). The survival ratio of cells which had been treated with Edaravone before the anoxic damage (Prevention-E-Damage-S group) was similar with that in Positive-S group ($p>0.05$), while the survival ratio of cells treated with Edaravone after the anoxic damage (Damage-S-Therapy-E group) was significant higher than Positive-S group ($p<0.05$) (Fig. 1B). Both cells treated with Edaravone before (Prevention-E-Damage-S group) and after (Damage-S-Therapy-E group) oxidative damage had a higher survival ratio ($p<0.05$, Fig. 1D).

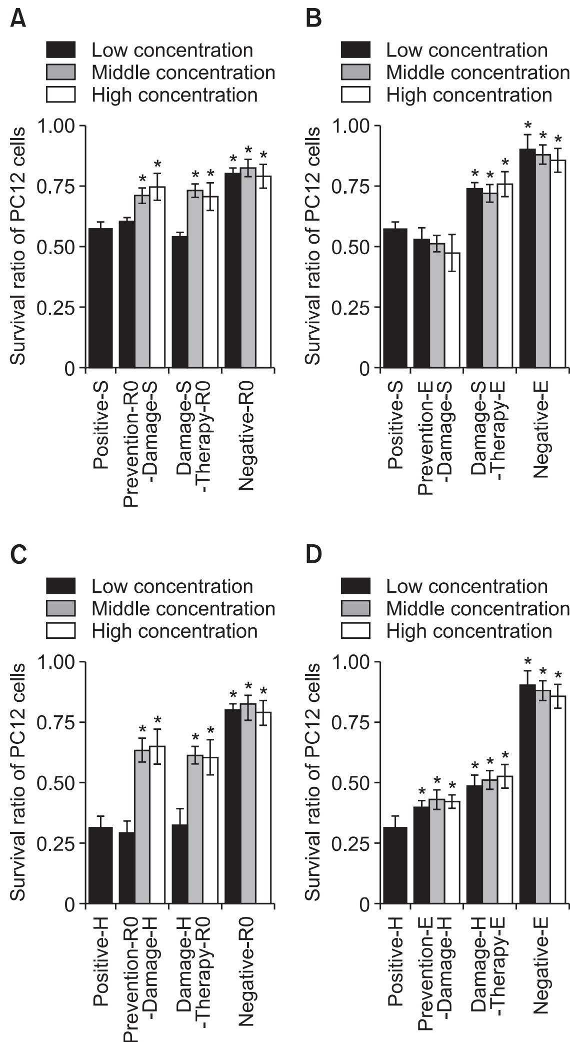

Fig. 1. Survival ratio of PC12 cells in different groups. With ginsenoside R0 (2.5 mg/ml, 7.5 mg/ml, and 12.5 mg/ml) or Edaravone (1.0 mg/ml, 1.25 mg/ml, and 1.5 mg/ml) treatment, survival ratio of PC12 cells was estimated. Panel (A) shows the comparison in Prevention-R0-Damage-S, Damage-S-Therapy-R0, Positive-S, and Negative-R0 groups. Panel (B) shows the comparison in Prevention-E-Damage-S, Damage-S-Therapy-E, Positive-S, and Negative-E groups. Panel (C) compares the survival ratio in Prevention-R0-Damage-H, Damage-H-Therapy-R0, Positive-H, and Negative-R0 groups. Panel (D) compares the survival ratio in Prevention-E-Damage-H, Damage-H-Therapy-E, Positive-H, and Negative-E groups. Data are expressed as means ± SD. Compared with control, *$p<0.05$.

The apoptosis of the cells

The apoptosis of each group of PC12 cells was tested by flow cytometry. In Fig. 2, we could see that there were very rare apoptosis in Negative-R0, Negative-E, Prevention-R0-Damage-S, Damage-S-Therapy-R0, Damage-H-Therapy-R0 and Prevention-R0-Damage-H groups. And the apoptosis was mainly present in Positive-S, Positive-H, Prevention-E-Damage-S, Damage-S-Therapy-E and Prevention-E-Damage-H groups.

MDA level

MDA expressions in PC12 cells and the cell cultured medium were tested to determine the damage to the cells. With sodium dithionite treatment, the expression of MDA of each

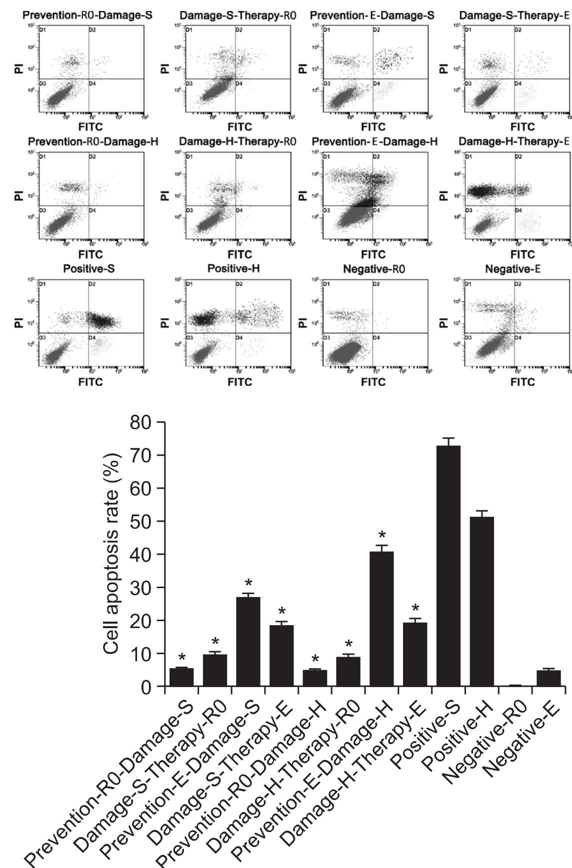

Fig. 2. Cell apoptosis in different groups by flow cytometry. With ginsenoside R0 (7.5 mg/ml) or Edaravone (1.5 mg/ml) treatment, apoptosis in PC12 cells was estimated. D1 quadrant stands for the necrotic cells, D2 quadrant stands for cells with late apoptosis, D3 quadrant stands for the normal alive cells, and D4 quadrant stands for cells with early apoptosis. Data are expressed as means ± SD. Compared with control, *$p<0.05$.

group in the cultured medium did not have much difference (p>0.05, Fig. 3A and 3B). In the PC12 cells, the MDA expression was significantly higher in the Positive-S group (p<0.05, Fig. 3A and 3B), MDA in Prevention-R0-Damage-S group was similar with the Negative-R0 group (p>0.05), while MDA in Damage-S-Therapy-R0, Prevention-E-Damage-S, and Damage-S-Therapy-E groups were higher than the negative controls (p<0.05, Fig. 3A), but still significantly lower than the positive control (p<0.05, Fig. 3A and 3B). With hydrogen peroxide treatment, MDA expressions of Positive-H group in the cultured medium was significantly higher than other groups (p<0.05, Fig. 3C and 3D) except Damage-H-Therapy-R0 group (p>0.05, Fig. 3C). Cellular MDA in Prevention-R0-Damage-H, Damage-H-Therapy-R0, Prevention-E-Damage-H, and Damage-H-Therapy-E groups were higher than the negative controls (p<0.05), but still significantly lower than the positive control (p<0.05) (Fig. 3C and 3D).

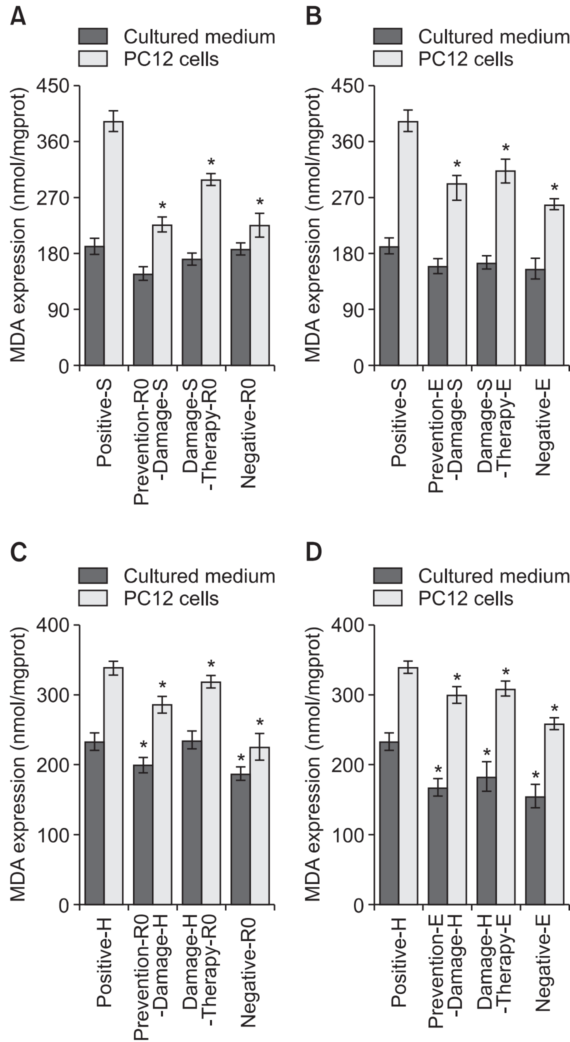

Fig. 3. MDA expression levels in PC12 cells and their cultured medium with different treatments. With ginsenoside R0 (7.5 mg/ml) or Edaravone (1.5 mg/ml) treatment, MDA expression levels were detected. Panel (A) shows the comparison in Prevention-R0-Damage-S, Damage-S-Therapy-R0, Positive-S, and Negative-R0 groups. Panel (B) shows the comparison in Prevention-E-Damage-S, Damage-S-Therapy-E, Positive-S, and Negative-E groups. Panel (C) compares the MDA expression levels in Prevention-R0-Damage-H, Damage-H-Therapy-R0, Positive-H, and Negative-R0 groups. Panel (D) compares the MDA expression levels in Prevention-E-Damage-H, Damage-H-Therapy-E, Positive-H, and Negative-E groups. Dark grey presents MDA expression levels in cultured medium, light grey presents MDA expression levels in PC12 cells. Data are expressed as means ± SD. Compared with control, *p<0.05.

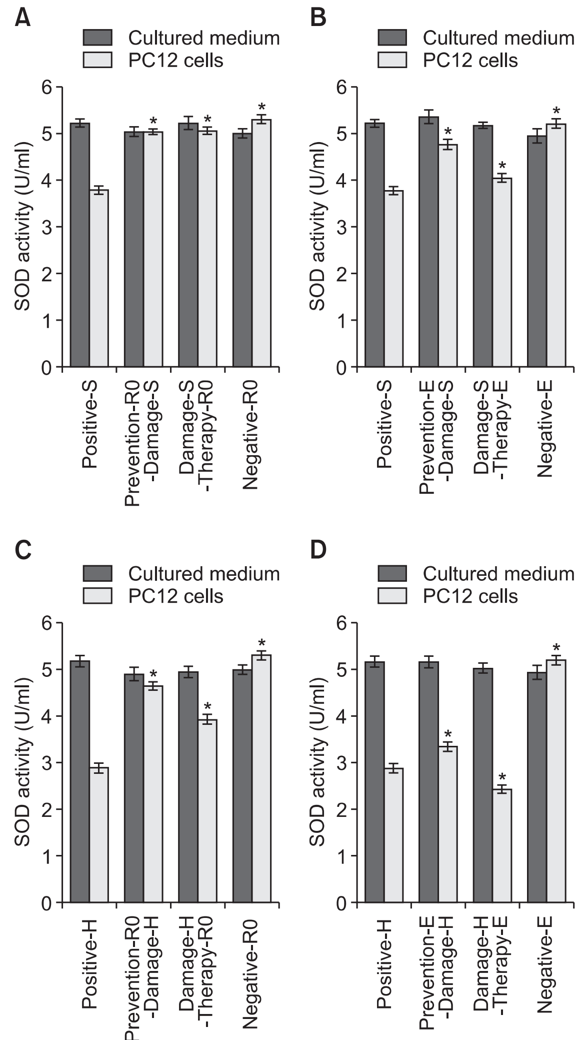

Fig. 4. SOD activities in PC12 cells and their cultured medium with different treatments. With ginsenoside R0 (7.5 mg/ml) or Edaravone (1.5 mg/ml) treatment, SOD activities were detected. Panel (A) shows the comparison in Prevention-R0-Damage-S, Damage-S-Therapy-R0, Positive-S, and Negative-R0 groups. Panel (B) shows the comparison in Prevention-E-Damage-S, Damage-S-Therapy-E, Positive-S, and Negative-E groups. Panel (C) compares the SOD activities in Prevention-R0-Damage-H, Damage-H-Therapy-R0, Positive-H, and Negative-R0 groups. Panel (D) compares the SOD activities in Prevention-E-Damage-H, Damage-H-Therapy-E, Positive-H, and Negative-E groups. Dark grey presents SOD activities in cultured medium, light grey presents SOD activities in PC12 cells. Data are expressed as means ± SD. Compared with control, *p<0.05.

SOD activity

SOD activity in PC12 cells and the cell cultured medium were tested to determine the anti-oxidant ability of the cells. Either with sodium dithionite or hydrogen peroxide treatment, the activity of SOD in each group in the cultured medium did not have much difference (p>0.05, Fig. 4). With sodium dithionite treatment, cellular SOD activity in Prevention-R0-Damage-S, Damage-S-Therapy-R0, and Negative-R0 was significantly higher than the Positive-S group (p<0.05), and there was no difference among these three groups (p>0.05) (Fig. 4A); cellular SOD activity in Prevention-E-Damage-S, Damage-S-Therapy-E, and Negative-E was significantly higher than the Positive-S group (p<0.05), and SOD in Damage-S-Therapy-E was lower than the other two groups (p<0.05) (Fig. 4B). With hydrogen peroxide treatment, cellular SOD activity in Prevention-R0-Damage-S, Damage-S-Therapy-R0, and Negative-R0 was significantly higher than the Positive-S (p<0.05), and Prevention-R0-Damage-S was higher than Damage-H-Therapy-R0 (p<0.05) (Fig. 4C). Cellular SOD activity in Prevention-E-Damage-H and Negative-E was higher than the Positive-H (p<0.05), but the SOD in Damage-H-Therapy-E was lower than the Positive-H (p<0.05) (Fig. 4D).

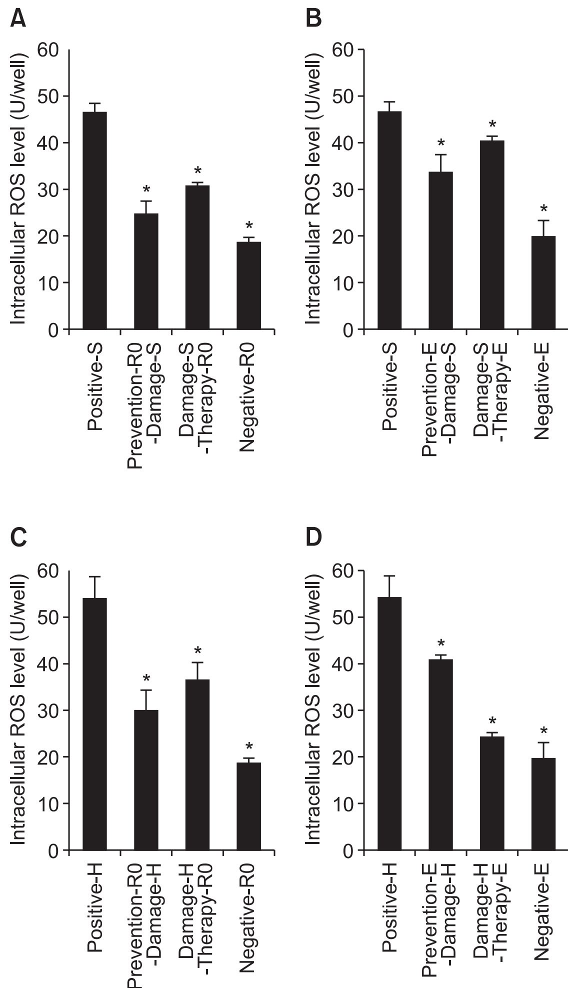

Fig. 5. Intracellular ROS level in PC12 cells with different treatments. With ginsenoside R0 (7.5 mg/ml) or Edaravone (1.5 mg/ml) treatment, intracellular ROS was detected in PC12 cells after sodium dithionite or hydrogen peroxide damage. Data are expressed as means ± SD. Compared with control, *p<0.05; Prevention-R0-Damage-S=pretreated with ginsenoside R0 and then treated with sodium dithionite, Damage-S-Therapy-R0=pretreated with sodium dithionite and then treated with ginsenoside R0, Prevention-E-Damage-S=pretreated with Edaravone and then treated with sodium dithionite, Damage-S-Therapy-E=pretreated with sodium dithionite and then treated with Edaravone, Prevention-R0-Damage-H=pretreated with ginsenoside R0 and then treated with hydrogen peroxide, Damage-H-Therapy-R0=pretreated with hydrogen peroxide and then treated with ginsenoside R0, Prevention-E-Damage-H=pretreated with Edaravone and then treated with hydrogen peroxide, Damage-H-Therapy-E=pretreated with hydrogen peroxide and then treated with Edaravone, Positive-S=only treated with sodium dithionite, Positive-H=only treated with hydrogen peroxide, Negative-R0=only treated with ginsenoside R0, Negative-E=only treated with Edaravone.

Intracellular ROS level

Sodium dithionite and hydrogen peroxide treatments significantly increased the intracellular level of ROS, especially the hydrogen peroxide. Pretreatment with ginsenoside R0 significantly inhibited the elevated intracellular level of ROS by sodium dithionite or hydrogen peroxide (p<0.05), while the pretreatment with Edaravone had less effect in the process against hydrogen peroxide damage than in sodium dithionite damage (p<0.05). Treated with either ginsenoside R0 or Edaravone after both damages significantly decreased the intracellular level of ROS (p<0.05; Fig. 5).

DISCUSSION

Most of the previous studies for ginsenosides were conducted *in vitro* and focused on neurons. Leung *et al.* studied the neuroprotective effects of ginsenoside Rg1 in primary nigral neurons against rotenone toxicity (Leung *et al.*, 2007). Considering that ginsenosides could not reach sufficient concentration in the neurons *in vivo*, PC12 cells were chosen for the *in vitro* study to investigate the effects of ginsenoside R0 under an anoxic or oxidative environment. This cell line was established from a rat transplantable rat adrenal pheochromocytoma, which respond reversibly to NGF (nerve growth factor) by induction of the neuronal phenotype, and is a useful model systems for neurobiological and neurochemical studies.

In our study, we first used 3 different concentrations of ginsenoside R0 and Edaravone to pretreat or treat PC12 cells after an anoxic or oxidative damage, and found the survival ratio increased when the concentration of ginsenoside R0 increased from 2.5 mg/ml to 7.5 mg/ml (p<0.05), but the survival ratio did not increase much when the concentration increased from 7.5 mg/ml to 12.5 mg/ml; the survival ratio was not so much effected by the changes of Edaravone concentration. We decided that the optimal concentrations to use in this experiment are 7.5 mg/ml of ginsenoside R0 and 1.5 mg/ml of Edaravone. In this study, we found that both the pretreatment and treatment with ginsenoside R0 would increase the survival ratio of PC12 cells when it underwent an anoxic damage by sodium dithionite or an oxidative damage by hydrogen peroxide; the treatment with Edaravone would also increase the survival ratio in the two damages respectively, however, the pretreatment with Edaravone did not get such a good survival ratio, especially in the anoxic damage, which was similar with

the Positive-S group (Fig. 1). The results of the apoptotic cells obtained from Flow cytometry supported the survival ratio (Fig. 2). It seems that the ginsenoside R0 had good preventive and therapeutic effects on either anoxic or oxidative damage to PC12 cells, while Edaravone had therapeutic effects on the both damages, but only had preventive effects on oxidative damages, and the therapeutic effects of Edaravone was not as good as ginsenoside R0. Our study showed a poorer protective and therapeutic effect of Edaravone than ginsenoside R0.

Therefore, we assumed that ginsenoside R0 might have an ability to protect cells from the anoxic and oxidative damages, just like some other members of ginsenosides, and its protective effect might due to the anti-oxidant function. To verify this hypothesis, MDA expression level and SOD activity were detected in PC12 cells and their cultured medium which were with different treatments of ginsenoside R0 and Edaravone. MDA is a main product of oxidative damage and SOD is an important antioxidant enzymes. It has been shown that SOD has the ability to transform superoxide anions to hydrogen peroxide. Elevated GSH level, SOD activity provide a repair mechanism for oxidized membrane components (Xiao *et al.*, 2008).

This study found that the treatment of PC12 cells with sodium dithionite caused a marked rise in oxidative stress as characterized by excessive MDA production and a reduction in SOD activity. MDA levels and SOD activities varied much in cells with different treatments, but not so much in the cultured medium. For either anoxic or oxidative damage treatment in PC12 cells, both ginsenoside R0 and Edaravone had a better effect on decreasing MDA level with pretreatment, and the preventive effects of ginsenoside R0 were better than Edaravone. For anoxic damage in PC12 cells, ginsenoside R0 had a better effect on maintaining a higher SOD activity than Edaravone, no matter with pretreatments or treatments, and pretreated Edaravone had a better effect than treated after damage. For oxidative damage in PC12 cells, Edaravone had no effect when treated before damage, and even decrease the SOD activity when treated after damage; while ginsenoside R0 still had a good maintaining effect on SOD activity. It suggested that the cell protective effect of ginsenoside R0 may be related to the antioxidant action.

CONCLUSION

In summary, ginsenoside R0 may protect against sodium dithionite or hydrogen peroxide induced cell death in PC12 cells better than Edaravone injection, preventive administration was more effective than the therapeutic treatment. The cell viability of anoxic or oxidative injured cells was increased in parallel with the increases in the SOD activity, and the decrease in MDA expression level, suggesting the neuroprotective effect of ginsenoside R0 was mediated by its antioxidant function. We believe that ginsenoside, its protection efficiency is higher than Edaravone, R0 can protective nerve cells by a different mechanism than that of Edaravone. The prevent ability of ginsenoside R0 is higher than its repair ability in neuronal cell protection. We believe the results obtained from this study would shed light on better understanding the pharmacological target and mechanisms of ginsenoside R0.

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