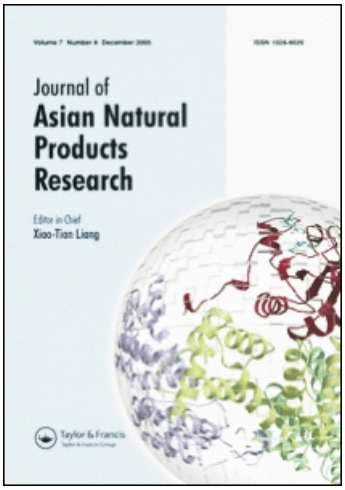

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ORIGINAL ARTICLE

Ginsenoside Rg1 promotes proliferation and neurotrophin expression of olfactory ensheathing cells

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Transplantation of olfactory ensheathing cells (OECs) is currently considered to be one of the most promising repair strategies for human spinal cord injury. However, the factors that regulate OECs are still poorly understood. Ginsenoside Rg1 (Rg1), the phytosterol from *Panax ginseng*, is a potent neuroprotective agent that promotes axonal regeneration. The aim of this study is to determine whether Rg1 would influence the biological activity of OECs. Primary cultured OECs from the olfactory bulb of neonatal rats were treated with Rg1 of various concentrations and durations. Using MTT and bromodeoxyuridine assays, we found that Rg1 significantly promoted cell proliferation, with an optimal concentration of 40 μg/ml of Rg1 at 72 h. In addition, RT-PCR and ELISA assays showed that Rg1 could upregulate the mRNA expression and secretion of glial cell-derived neurotrophic factor, brain-derived neurotrophic factor, and nerve growth factor. These results suggest that Rg1 may have a great potential in OEC therapy.

Keywords: olfactory ensheathing cells; ginsenoside Rg1; proliferation; brain-derived neurotrophic factor; glial cell-derived neurotrophic factor; nerve growth factor

1. Introduction

Evidence suggests that transplanted olfactory ensheathing cells (OECs) may improve the functional deficits associated with injuries to the spinal cord [1], possibly via their production of a variety of neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) [2]. Therefore, understanding what regulates OECs and obtaining data about clinically relevant regulation agents would be extremely valuable.

Panax ginseng has been used in Asia as an immunomodulatory and anti-aging tonic for thousands of years, and its phytosterol, ginsenoside Rg1 (Rg1, $C_{42}H_{72}O_{14}$; Figure 1(A)), is one of its major active ingredients. Previous studies have indicated that ginsenosides can promote the proliferation of Schwann cells, which are a type of peripheral glial cells [3]. Moreover, Rg1 has been shown to promote axonal regeneration and protect neurons from lipid oxidation and apoptosis [4]. In addition, as a phytosterol, the chemical structure of Rg1 is very similar to that of a glucocorticoid (Figure 1(B)),

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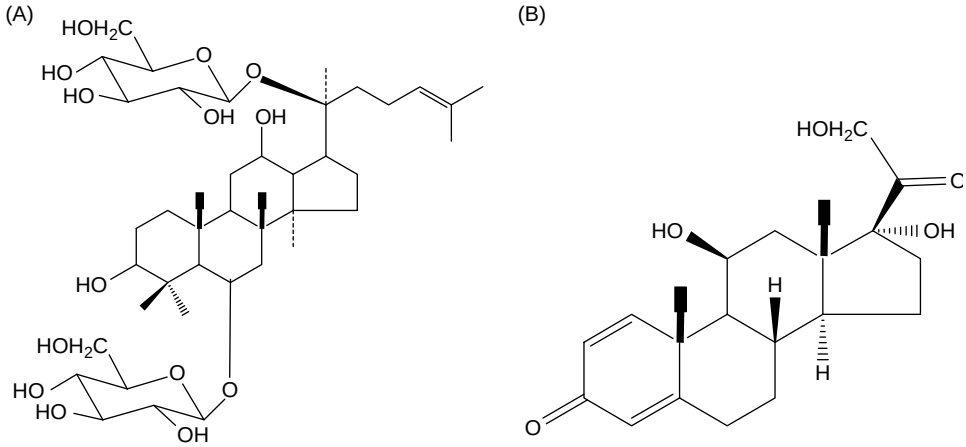

Figure 1. (A) Structure of Rg1. (B) Structure of glucocorticoid (methylprednisolone).

which regulates the phenotypic activity of glial cells [5]. Consequently, this study is designed to determine whether Rg1 would affect OECs.

2. Results and discussion

The OECs were identified as specifically expressing NGFRp75 (Figure 2(A),(B)), and the percentage of purified cells in the cultures was 90–93%. The OD value corresponds to the cell number and viability in the MTT assay. The cell number reached the maximum on 20 and 40 μg/ml Rg1 groups at 72 h, and there was no significant difference between them (0.377 ± 0.006 vs. 0.386 ± 0.016, $n = 5$, $p > 0.05$). However, the cell number of the 40 μg/ml group was significantly higher than that of the control, 5 and 10 μg/ml groups since 48 h (0.348 ± 0.017 vs. 0.299 ± 0.012, 0.312 ± 0.014, and 0.315 ± 0.012, $n = 5$, $p < 0.05$). Moreover, the OD value of the 40 μg/ml group at 72 h was significantly higher than that of the 24 and 48 h groups (0.386 ± 0.016 vs. 0.311 ± 0.018 and 0.348 ± 0.017, $n = 5$, $p < 0.05$) (Table 1). Therefore, Rg1 promoted the proliferation and viability of OECs, and the effect was dose- and time-dependent with the optimal effect on 40 μg/ml at 72 h. Comparing with the control group, the OEC proliferation index was also significantly increased by Rg1 in the bromodeoxyuridine (BrdU) assay (9% vs. 17%, $n = 3$, $p < 0.05$) (Figure 2(C)), which corresponded to that of the 2 μM forskolin group (17% vs. 20%, $n = 3$, $p > 0.05$) (Figure 2(C)).

In addition, Rg1 significantly upregulated the mRNA expression of GDNF, BDNF, and NGF, as shown by RT-PCR ($n = 3$, $p < 0.05$) (Figure 3). Moreover, cell number-adjusted ELISA data demonstrated that Rg1 increased the secretion of GDNF from 76 ± 19.1 to 111.8 ± 10.2 (pg/ml), BDNF from 428.8 ± 56.5 to 954.6 ± 82.2 (pg/ml), and NGF from 206.2 ± 33.5 to 348.5 ± 46.7 (pg/ml) ($n = 3$, $p < 0.05$) (Figure 4).

Cell transplantation is becoming one of the most promising strategies for repairing human spinal cord injury. This technique helps to replace lost or damaged neurons and induce axonal regeneration, provides a source of cells to facilitate remyelination, and delivers neurotrophics to promote cellular protection and plasticity [6]. OECs are a specialized type of glial cell from the olfactory system, and they are considered to be the most promising cells for transplantation [7] because they

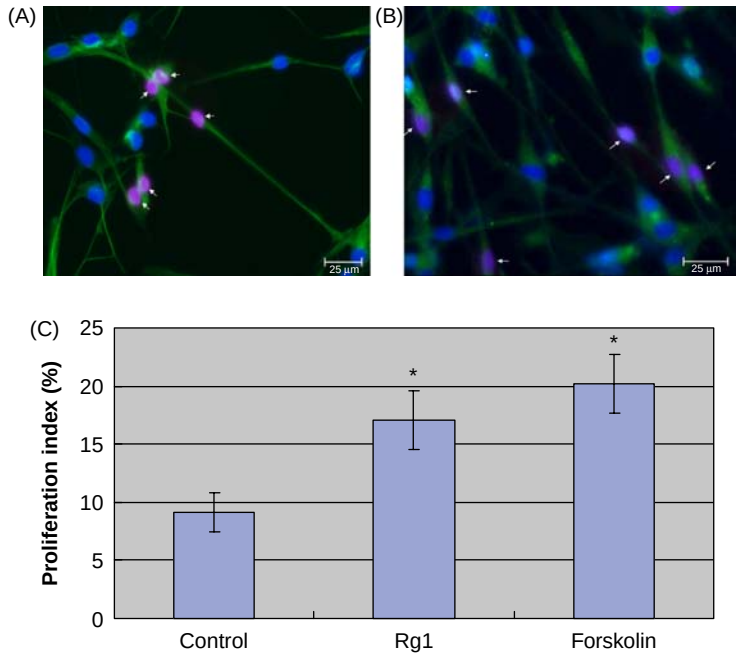

Figure 2. (A), (B) Immunofluorescent staining for BrdU (red) and p75 (green) visualized under a fluorescence microscope. H33258 marked all of the nuclei (blue). Arrows indicate the dividing cells [(A) Rg1 group, (B) forskolin group]. (C) The OEC proliferation indices of the control, Rg1, and forskolin-treated groups are 9, 17, and 20%, respectively. $*p < 0.05$, as compared with the control group ($n = 3$) (color online).

possess biological activities of both central astrocytes and peripheral Schwann cells. The ensheathing cell transplants can secrete neurotrophins such as GDNF, BDNF, and NGF to create a beneficial environment for axon regeneration and remyelination and reduce glial scars and cavities [8]. However, a main concern about this approach lies in the limited source and unabiding viability of the cells, because OECs can now only be obtained from primary culture, and the proliferation activity and vitality of the cells decreased progressively during culture and passage. Previous studies have confirmed that some chemocytokines, such as basic fibroblast growth factor (bFGF), neurotrophin 3, and lysophosphatidic acid, promote the cell proliferation *in vitro* [9–11], but these factors are unsuitable or unsafe for clinical trial.

P. ginseng is known to have a wide spectrum of medicinal effects, such as tonic, immunomodulatory, anti-mutagenic, adaptogenic, and anti-aging activities [12]. Rg1 (Figure 1(A)), a panaxatriol ingredient, is a most prevalent active constituent of ginseng root [4,13,14]. Both *in vitro* and *in vivo* experiments have demonstrated that Rg1 not only promotes the proliferation of functional neurons, but also protects neurons in ischemic situations [4,13,14]. In addition, Rg1 can significantly enhance the survival of dopaminergic neurons and increase the number and length of neurites in glutamate-induced apoptosis conditions in the process of neurological recovery [15]. Based on these clues, we investigated whether Rg1 could affect OECs. If Rg1 has an effect on OECs, it might serve as a clinically available regulating agent for OECs.

Table 1. Effect of Rg1 treatment on OECs determined by the MTT assay (measured as mean ± SD).

Time (h)	Control	5 μg/ml	10 μg/ml	20 μg/ml	40 μg/ml	80 μg/ml
24	0.278 ± 0.031	0.290 ± 0.025	0.299 ± 0.042	0.305 ± 0.012	0.311 ± 0.018	0.302 ± 0.022
48	0.299 ± 0.012	0.312 ± 0.014	0.315 ± 0.012	0.326 ± 0.014	0.348 ± 0.017[a,b]	0.327 ± 0.023
72	0.307 ± 0.010	0.322 ± 0.017	0.346 ± 0.018	0.377 ± 0.006[a,b,c]	0.386 ± 0.016[a,b,c]	0.360 ± 0.025[a]
96	0.302 ± 0.019	0.324 ± 0.020	0.345 ± 0.021	0.369 ± 0.027[a]	0.375 ± 0.017[a]	0.349 ± 0.025

Notes: [a] $p < 0.05$, vs. the control group.
[b] $p < 0.05$, vs. 5 and 10 μg/ml groups for the same time.
[c] $p < 0.05$, vs. 24 and 48 h groups at the same concentration.

The experiments first showed that Rg1 has an effect on OECs, and its optimal dose and treatment time were determined. The MTT assay results showed that Rg1 promoted the proliferation and viability of the cells. Furthermore, the BrdU result for Rg1 was consistent with the MTT result. To further explore the effect of Rg1 on the cells, neurotrophin-producing functions were investigated using RT-PCR and ELISA. These techniques confirmed that Rg1 significantly increased the mRNA expression and protein secretion of GDNF, BDNF, and NGF. Therefore, the other effects of the Rg1 molecule (e.g. its protective effect, migration stimulation, and chemotaxis) on OECs warrant further study. Moreover, its potency *in vivo* needs to be explored.

At present, studies on this phytosterol derived from herbal medicine are few, so its possible mechanisms of action remain unclear. However, given its estrogen-like and glucocorticoid-like properties [16,17] and the steroidal skeleton of the molecule, several clues exist. (1) The estrogen-like actions of Rg1 might be mediated through crosstalk between the estrogen receptor and the growth factor receptor-dependent special signaling pathways [16]. (2) As a functional ligand of the glucocorticoid receptor, Rg1 can directly conjugate to the receptor to activate a glucocorticoid-responsive element containing the luciferase reporter gene [18]. (3) Based on the estrogen receptor expression of the ensheathing cells [17], we speculate that Rg1 activates the estrogen receptor through the above-mentioned special signaling pathways; moreover, it might activate the mitogen-activated protein kinase pathway and intracellular calcium release [19,20].

The present study confirms that Rg1 is a potent regulating agent of OECs. It promotes cell proliferation as well as mRNA expression and protein secretion of GDNF, BDNF, and NGF. Nevertheless, more experiments are needed to obtain a comprehensive understanding of the role

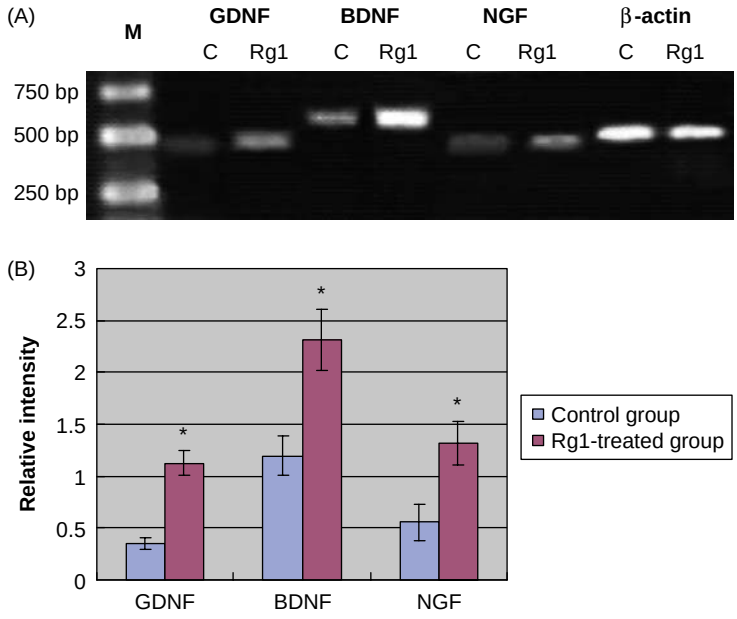

Figure 3. (A) RT-PCR products of GDNF, BDNF, and NGF in the control and Rg1 groups stained on a 2% agarose gel. (B) The mRNA expression for GDNF, BDNF, and NGF relative to β-actin in the control and Rg1 groups (mean ± SD). $*p < 0.05$, as compared with the control group ($n = 3$).

of Rg1 on the biological activities of OECs and its mechanisms of action.

3. Materials and methods

3.1 OEC culture and immunoidentification

The protocols for animal study were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Soochow University. The OECs were primarily cultured and purified using differential adherent velocity combined with cytosine arabinoside (Ara-c; Sigma, St Louis, MO, USA). Briefly, 1–3-day-old neonatal rats were anesthetized with 10% chloraldurat (350 mg/kg) and decapitated. The olfactory bulbs were then detached,

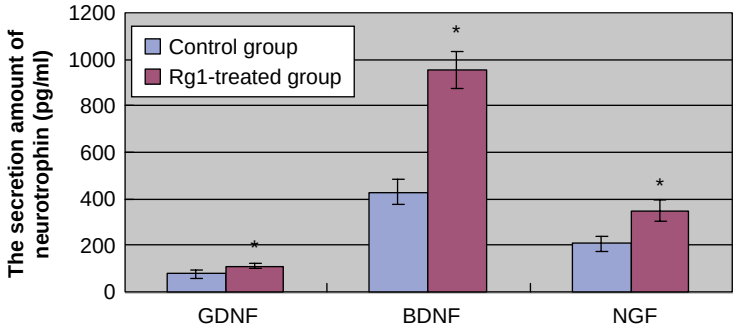

Figure 4. Effect of Rg1 on GDNF, BDNF, and NGF secretion from OECs. Culture supernatants (72 h) were collected and determined by ELISA kits (Promega, Madison, WI, USA). $*p < 0.05$ ($n = 3$), as compared with the control group. Data were adjusted by: original ELISA data (pg/ml)/proliferation coefficient (proliferation coefficient = post-intervention cell number/pre-intervention cell number).

and the olfactory nerve and glomerular layers were dissected from the rest of the bulbs and minced. After digestion with 2.5 g/l trypsase (Gibco® Invitrogen, Grand Island, NY, USA) at 37°C for 10 min, the isolated cells were centrifuged and resuspended in DMEM/F-12 medium (Gibco® Invitrogen) containing 10% fetal bovine serum (FBS; Sigma). The cell suspension was seeded into uncoated flasks and incubated (37°C/5% CO_2/humidified) for 24 h, during which the vast majority of contaminating cells, such as fibroblasts, adhered more readily to the uncoated flasks. The supernatant, which mostly contained OECs, was transferred into poly-L-lysine (PLL; Sigma)-coated flasks. After incubation for another 24 h, the culture was treated with 2 mg/l Ara-c for 36 h to further minimize the population of contaminating cells. Purified OECs were cultured in DMEM/F-12/10% FBS before intervention with Rg1, and samples of each batch of the purified cells were identified by NGFRp75 immunofluorescent staining. Briefly, cells were fixed overnight at 4°C in 40 g/l paraformaldehyde (Sigma), and then incubated in rabbit anti-NGFRp75 (1:200; Sigma) for 90 min, followed by fluorescein isothiocyannate-conjugated goat anti-rabbit IgG antibody (1:70; Sigma) for another 90 min, at room temperature. Immediately, Hoechst 33258 (Sigma) was added to mark all of the nuclei. OECs were identified as positive for both NGFRp75 and Hoechst 33258.

3.2 MTT assays

Purified OECs were harvested by trypsinization, counted, and seeded (2000 cells/well) into 30 wells of a 96-well plate, and the 30 wells were divided into six groups (5 wells/group). Cells were cultured in N_2 (Gibco® Invitrogen) serum-free medium for 24 h until all cells are attached to the bottom of the well. Rg1 (98%; National Administration of Pharmaceutical and Biological Products, Beijing, China) in a concentration gradient of 0 (control), 5, 10, 20, 40, and 80 μg/ml (diluted in DMEM/F-12) was then added to the wells of the corresponding six groups, and four plates were exposed to Rg1 for 24, 48, 72, and 96 h, respectively. MTT (Sigma, 5 mg/ml in PBS) of 10 μl/well was added to the cultures for the last 4 h of treatment. The culture medium was then replaced with DMSO (Sigma) of 150 μl/well to dissolve the purple crystals. Finally, the absorbance was read at OD 570 nm, which correlates with cell number and viability [21].

Based on the results of the MTT assays, we concluded that Rg1 at a concentration of 40 μg/ml and 72 h would be optimal for OEC culture (Table 1). Therefore, these parameters were used in the following experiments.

3.3 BrdU assay of cell proliferation

Cell proliferation was measured by detecting the incorporation of BrdU (Sigma). Forskolin (Sigma), known as OEC mitogen, was used as a reference to further evaluate the proliferation effect of Rg1 [22]. Purified OECs were seeded onto coverslips and cultured in a N_2 serum-free medium for 24 h. The cells were then divided into three groups: one of which was exposed to 40 μg/ml Rg1, one to 2 μM forskolin, and one to a medium only as a blank control. They were incubated for 72 h. To label the nuclei-synthesizing DNA, 10 μmol/ml BrdU was added to cultures for the last 24 h before staining. The cells were fixed with 4% paraformaldehyde for 30 min, treated with 1 and 2 M HCl for 10 min to denature the DNA, and then treated with 0.1 mol/l borate to counteract the acid. Thereafter, the cells were incubated overnight at 4°C in mouse anti-BrdU monoclonal antibody (1:1000; Chemicon, CA, USA), and then stained with phycoerythrin-conjugated goat anti-mouse IgG antibody (1:70; Sigma) for 1 h at 37°C. Subsequently, immunostaining

of p75 and H33258 was performed as described above. Five independent fields were scanned under a fluorescence microscope or at least 400 cells/coverslip were counted to calculate the proliferation rate. The cell proliferation index (%) was calculated as follows: (no. of BrdU+ and p75+)/(no. of p75+) × 100%.

3.4 RT-PCR and ELISA for GDNF, BDNF, and NGF

Purified cells were counted (pre-intervention cell number) and plated into 25 cm^2 PLL-coated flasks and cultured in a N_2 serum-free medium. The experimental cells were then treated with 40 μg/ml Rg1 for 72 h, and the control cells were retained in the medium for the same period of time. The culture media were harvested for ELISA assays and cells were trypsinized for RT-PCR; meanwhile, trypsinized cells were counted thrice with a hemacytometer (post-intervention cell number). The ELISA data were adjusted by cell number variation due to the effect of Rg1 on cell proliferation. The secreted level of neurotrophins was regulated as follows: original ELISA data (pg/ml)/proliferation coefficient (proliferation coefficient = post-intervention cell number/pre-intervention cell number)

Total RNA was isolated using Omega reagent (Omega, Irvine, CA, USA) and RT-PCR was performed following the instructions of the TaKaRa kit (TaKaRa, Dalian, China). DNA primers were designed from the following sequences: β-actin – sense 5′-GTCCCTGTATGCCTCTGGTC-3′, anti-sense 5′-GGTCTTTACGGATGTCAACG-3′ (456 bp); GDNF – sense 5′-GATGAAGTTATGGGATGTCG-3′, anti-sense 5′-TTCCTCCTTGGTTTCGTAG-3′ (418 bp); BDNF – sense 5′-TGGTTATTTCATACTTCGGTTGC-3′, anti-sense 5′-ATGGGATTACACTTGGTCTCG-3′ (550 bp); NGF – sense 5′-GTTTAGCACCCAGCCTCC-3′, anti-sense 5′-CCTCTTCTTGCAGCCTT CC-3′ (459 bp). The conditions for RT-PCR were as follows: denaturation at 94°C for 2 min followed by 28 cycles at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. The PCR products were analyzed after electrophoresis on a 2% agarose gel and staining with ethidium bromide. The relative intensities of the bands were analyzed using SmartView 2001 software (Furi Inc., Shanghai, China). Simultaneously, the culture medium was collected to detect the protein content of GDNF, BDNF, and NGF using ELISA kits (Promega, Madison, WI, USA) according to the manufacturer's instructions.

3.5 Statistical analysis

All data were the result of at least three separate experiments and are expressed as mean ± SD. Statistical analysis was performed using SPSS® version 11.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was evaluated by Student's *t*-test and defined as $p < 0.05$.

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