Effects of ginsenoside-Rg1 on the proliferation and glial-like directed differentiation of embryonic rat cortical neural stem cells *in vitro*

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Abstract. Ginsenoside-Rg1, the main active component of *Panax notoginseng*, exhibits a number of pharmacological functions, including promoting protein synthesis in the brain, increasing the number of synapses, improving memory and promoting recovery of brain function following injury. The effect of ginsenoside-Rg1 on proliferation and glial-like-directed differentiation in the cortical neural stem cells (NSCs) of embryonic rat brain was investigated. The present study used MTS assays to identify the optimum dose and window time of ginsenoside-Rg1 administration to stimulate the proliferation of cortical NSCs in the rat embryonic tissue. The oxygen glucose deprivation (OGD) set-up was used as a cell injury model. Immunofluorescent staining was used for identification of NSCs and subsequent observation of their proliferation and glial-like directed differentiation. Nestin expression was the marker for the presence of NSCs among the cortical cells of embryonic rat brain. The optimum dose of ginsenoside-Rg1 for proliferation of NSCs was 0.32 μg/ml. The optimum window time of 0.32 μg/ml ginsenoside-Rg1 administration on proliferation of NSCs was 6 h. Ginsenoside-Rg1 at 0.32 μg/ml concentration promoted incorporation of bromo-2-deoxyuridine, and expression of nestin and vimentin in primary and passaged NSCs, and NSCs following OGD. Ginsenoside-Rg1 had a role in promoting proliferation and glial-like-directed differentiation of cortical NSCs. The plausible explanation for these responses is that ginsenoside-Rg1 acts similarly to the growth factors to promote the proliferation and differentiation of NSCs.

Introduction

In traditional theory, neurons of the central nervous system (CNS) are considered to be non-renewable and any injury to these cells is considered irreversible. If these neurons are damaged, they cannot produce new neurons to repair injury. This theory results in inhibition and distress in the prevention and treatment of injury, and degenerative diseases of the central nervous system (1). The discovery of neural stem cells (NSCs) contradicted this hypothesis (2,3). NSCs have the capacity to self-renew, proliferate, migrate and differentiate, and can differentiate into all cell types contained within the brain and spinal cord tissue, including neurons, astrocytes and oligodendrocytes (4). NSCs in the brain can be observed in two regions: The subventricle zone (SVZ) of lateral ventricles and subgranular zone of the hippocampal dentate gyrus (5,6). NSCs are also observed in the cortex (7), hippocampus (8), striatum (9) and other parts of the brain (10-12). Under normal conditions, NSCs are in a resting state. Injury stimulates these cells to replicate, proliferate and differentiate into new neural cells and replace necrotic cells. NSCs are essential for the formation of new neural circuits, and promote the repair of the structure and function of the brain following damage (13,14).

There are two intervention strategies involving NSCs for the treatment of injury and degenerative diseases of the CNS. The first type involves the promotion of endogenous NSC proliferation and differentiation (15,16). A recent study on this type of treatment has indicated that the number of

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endogenous NSCs was not sufficient to repair the lesion sites (17). The second type of intervention involves the exogenous transplantation of NSCs to the lesion sites (18,19). NSCs from the transplantation can survive, replicate and differentiate into local nerve cells. As a result, NSCs can repair the sites of injury (20,21). One of the aims of the future research in the field of structural repair of brain damage is the identification of drugs and methods that can stimulate proliferation, directional migration and differentiation of NSCs, through the precise isolation, culturing and identification of NSCs.

Renshen (*Panax ginseng* C. A. Mey) comes from the roots of *Araliaceae Panax*, first recorded in the 'Shen Nong's Herbal Classic', one of the important ancient books of traditional Chinese Medicine (22). Ginsenoside-Rg1 is the main active component of a plant species *Panax notoginseng*. Ginsenoside-Rg1 has a broad range of pharmacological functions. Ginsenoside-Rg1 has been demonstrated to increase the proliferating ability of neural progenitor cells, thus promoting the process of neurogenesis (23,24). Ginsenoside-Rg1 induces a neuroprotective effect against brain ischemia by inhibiting Ca^{2+} influx into primary cultured hippocampal neurons (25). In addition, ginsenoside-Rg1 promotes neuroprotective effects following intracerebral hemorrhage through anti-oxidation (26), reduces nerve cell damage, promotes protein synthesis in the brain, increases the number of synapses, improves memory and promotes recovery of brain function following injury (27-30). The authors previously demonstrated that ginsenoside-Rg1 can promote expression of proliferating cell nuclear antigen (PCNA), nestin, bromo-2-deoxyuridine (BrdU) incorporation, and the survival and self-renewal of cells in the SVZ of the lateral ventricle in the adult rat brain following cortical devascularization (31). In the present study, the cortical NSCs of embryonic rat brain were isolated, cultured and identified, to study the effect of ginsenoside-Rg1 on the survival, self-renewal and glial-like-directional differentiation of NSCs *in vitro*.

Materials and methods

Materials. A total of four female Sprague Dawley (SD) rats (weighing 280±10 g) at day 17 of gestation from the Genetic Institute of the Chinese Academy of Medical Sciences (Beijing, China; certificate of conformity, SCXK 2011-0004) were used for all experiments. All procedures in the animal experiments followed the instructions for the care and use of animals provided by the Beijing University of Chinese Medicine (Beijing, China). All procedures in the present study were carried out in accordance with institutional guidelines and approved by the Ethics Committee of Beijing University of Chinese Medicine; all surgeries were performed under anesthesia, and all efforts were made to minimize suffering. Ginsenoside-Rg1 (purity >99%, purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) was used as a stock solution for subsequent dilutions.

Antibodies: anti-nestin (cat. no. ab11306; Abcam, Cambridge, CB, UK), anti-BrdU (cat. no. ab8152; Abcam), anti-vimentin (cat. no. ab8978; Abcam), anti-Cy3-conjugated

Affinipure goat anti-rabbit immunoglobulin (Ig) G (cat. no. SA00009-2; ProteinTech, Wuhan, Hubei, P.R.C), fluorescein isothiocyanate (FITC)-conjugated Affinipure goat anti-mouse IgG (cat. no. SA00003-1; ProteinTech).

Primary culture and passage of cortical NSCs of embryonic rat brain. Cortical tissue of E17 embryonic rats was harvested under sterile conditions, the meninges were removed, and the cortex was washed in D-Hanks' solution (HyClone; GE Healthcare, Logan, UT, USA) under an anatomical microscope. The cortex was subsequently cut into 1-mm³ pieces in Dulbecco's modified Eagle's medium (DMEM)/F12 and then incubated at 37°C for 25 min in a 0.125% trypsin solution to digest the cortical tissue. The solution was then neutralized by the addition of 10% fetal bovine serum (HyClone; GE Healthcare). Single cell suspensions were prepared by repeated pipetting. Viable cells stained by trypan blue assay for 3 min at 37°C were then counted with light microscope (magnification, x40) and the cell number was adjusted to $1x10^6$ cells/ml culture medium. The cell suspensions were placed in 25-cm² flasks coated with poly-lysine (5 ml/flask) and placed in a cell incubator at 37°C containing 5% CO_2.

Following the formation of spheres in primary culture, clones were mechanically isolated and passaged in culture to form a single cell suspension at a density of $1x10^5$ cells/ml. Thereafter, clones were isolated mechanically and passaged every 5-7 days, using the above method and medium.

Screening for the optimal dose of ginsenoside-Rg1 for maximized proliferation of NSCs using the MTS method. NSCs were collected by centrifugation at 4°C (800 x g for 10 min), and single cell suspensions were prepared by mechanical pipetting and separating. NSC solutions were adjusted to densities of $1x10^5$ cells/ml and 100 μl/well was seeded into a 96-well culture plate, using serum-free medium [DMEM/F12, basic fibroblast growth factor (bFGF); Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA] and B27 (Invitrogen; Thermo Fisher Scientific, Inc). Groups, including the control group and ginsenoside-Rg1 groups (concentrations used were as follows: 0.001, 0.004, 0.016, 0.064, 0.32, 0.4, 2, 4, 8 and 16 μg/ml), were cultured for 3 days and 8 repeats were used for each group. A total of 3 h prior to the termination of culture experiment, 20 μl MTS/phenazine methosulfate (PMS) solution was added to each well and the solutions were cultured at 37°C for another 3 h. The optical density (OD) value was measured at a wavelength of 570 nm.

Screening for the optimum in vitro incubation time of NSCs in the ginsenoside-Rg1 solution using the MTS method. NSCs were collected by centrifugation at 4°C (800 x g for 10 min), and single cell suspensions were prepared by pipetting and separating. Using the optimum dose of ginsenoside-Rg1 identified in the preceding experiment, NSCs were seeded at $1x10^5$ cells/ml serum-free medium (DMEM/F12 and B12) in a 96-well culture plate (100 μl/well). The time points tested included 1, 3, 6, 12, 24, 48 and 72 h. NSC proliferation was verified at each time point and compared between the control and experimental groups. NSCs were cultured for different time points and 20 μl/well MTS/PMS solution was added

3 h prior to the end of each experiment. The OD value was measured at a wavelength of 570 nm.

BrdU labeling and immunofluorescence staining. BrdU was added into cell culture medium at a final concentration of 6 μg/ml. The cells were cultured for 4 days prior to the immunofluorescent staining. After 4 days of incubation under the described conditions, immunofluorescent staining using nestin, BrdU and vimentin was performed in order to analyze cell proliferation and differentiation. Antibodies against nestin, BrdU and vimentin were used to identify NSCs or NSC precursors and immature glial cells, respectively.

NSCs ($1x10^5$/ml, 500 μl) were plated onto coverslips coated with poly-D-lysine and immunofluorescent labeling for nestin/BrdU or nestin/vimentin was carried out for 6 h. The primary antibodies were nestin (1:500), nestin/BrdU (1:500 and 1:400 respectively), and nestin/vimentin (1:500 and 1:400, respectively), and incubated at 4°C overnight. Secondary antibodies were Cy3-conjugated affinipure goat anti-rabbit IgG (1:30) and FITC-conjugated affinipure goat anti-mouse IgG (1:30), and incubated in dark place at 4°C for 30 min.

Oxygen glucose deprivation (OGD). Cells were washed twice with D-Hanks' solution following culture for 9 or 10 days, and then the medium was replaced with Earle's balanced salt solution (HyClone; GE Healthcare) with or without glucose. The cells were randomly divided into the following three groups: The control group incubated in Earle's solution containing glucose under normoxic conditions; and the OGD group in which medium was replaced with glucose-free Earle's solution. These cells were placed in a hypoxic chamber, and treated with a gas mixture consisting of 7% CO_2 and 93% N_2 for 30 min to achieve an equilibrium, and then cultured in hypoxia for another 6 h. OGD was terminated by replacing the medium with one containing glucose and returning the cells back to a normoxic incubator. In the final group, the cells were treated as the OGD group above and additionally, ginsenoside-Rg1 was added at a dose of 0.32 μg/ml. Ginsenoside-Rg1 was maintained following OGD for 6 h at 37°C in an incubator containing 5% CO_2.

Image capture and statistical analysis. A total of three sections were randomly taken from each sample and three visual fields were randomly selected from each section. Area density, optical density and the number of positive cells were analyzed using Image Pro Proplus (version 6.0; Media Cybernetics, Inc., Rockville, MD, US). All results are presented as the mean ± standard deviation. The significance of variables was determined using one-way analysis of variance followed by the Bonferonni correction. Statistical analysis was performed using SPSS software (version 11.5; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Growth of cortical NSCs of the embryonic rat brain. NSCs proliferated to form spheres (primary cloning) 24 h following the primary culture with neural stem cell culture medium (DMEM/F12, bFGF and B27). An increase in the number of cells was observed after 7 days. The NSCs remained in a suspension and exhibited spherical shape and no neurite outgrowth (Fig. 1A and B). A large number of second generation clones were formed in the passage culture and the subcloning culture process is similar to the primary culture (Fig. 1C and D).

Identification of cortical NSCs in the embryonic rat tissue. Nestin was expressed in the cytoplasm of the cultured NSC cells. Positive cells exhibited a round or oval shape, certain cells exhibited symptoms of neuritis. Nuclear areas were nestin-negative and frequently positioned to one side of the cell body (Fig. 1E-H).

Optimum dose of ginsenoside-Rg1 on the proliferation of NSCs cultured for 3 days. The OD value increased significantly following treatment with 0.32 μg/ml ginsenoside-Rg1 measured by MTS compared with the control group (P<0.01; Fig. 2). Therefore, the dose of 0.32 μg/ml ginsenoside-Rg1 was the optimum dose for the proliferation of NSCs cultured for 3 days.

Optimal duration for treatment with 0.32 μg/ml ginsenoside-Rg1 for the proliferation of NSCs. Compared with the control groups, the OD value of NSCs treated with 0.32 μg ml^{-1} of ginsenoside-Rg1 increased significantly 6 h post initial treatment (P<0.01; Fig. 3). Therefore, the optimal duration of incubation with 0.32 μg/ml ginsenoside-Rg1 for the proliferation of NSCs was 6 h.

Effect of ginsenoside-Rg1 on the proliferation of cortical NSCs from embryonic rats. BrdU and nestin were co-expressed in the cortical NSCs of the control, OGD and ginsenoside-Rg1 groups. Nestin-positive cells were round or oval shaped and could be further divided into two kinds of cells: Those with and without neuritis. Nuclear areas were negative and frequently positioned to one side of the cell body. BrdU fluorescence in the nucleus of cultured cells and the cell body of positive cells was low. Nestin/BrdU co-expressing cells exhibited different sizes and shapes with extensions (Fig. 4). All types of positive cells decreased visibly in number in the OGD group and increased in the ginsenoside-Rg1 group. Statistical analysis of the data revealed that, compared with the OGD group, the area density, optical density, and the number of nestin- or BrdU- positive cells and nestin/BrdU double-positive cells, increased significantly in the ginsenoside-Rg1 group (all P<0.05; Figs. 5-7).

Effect of ginsenoside-Rg1 on glial-like-directed differentiation of cortical NSCs in embryonic rats. Vimentin and nestin were expressed and co-expressed in the control, OGD and treatment groups. Vimentin was expressed in the cytoplasm of NSCs towards one side of the cell but not in the nucleus. Cell bodies of vimentin-positive cells in the ginsenoside-Rg1-treatment group were round or oval-shaped, and could be further divided into large, medium and small. Cells exhibited single and multiple processes, which were woven into a mesh or parallel arrangement. Nuclear areas of cytoplasmic vimentin-positive cells were negative and
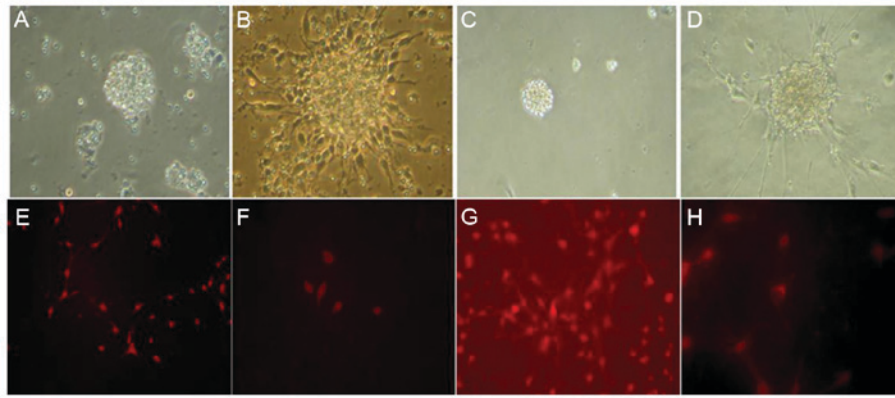
Figure 1. Representative images of cortical NSCs and nestin-positive cortical NSCs. (A) Primary culture of cortical NSCs (magnification, x200). (B) Primary adherent culture of cortical NSCs (magnification, x200). (C) Cloning of cortical NSCs in passage culture (magnification, x200). (D) Passage adherent culture of cortical NSCs (magnification, x200). (E) Nestin-positive cells of primary culture of cortical NSCs (magnification, x100). (F) Nestin-positive cells of primary culture of cortical NSCs (magnification, x200). (G) Nestin-positive cells of passage culture of cortical NSCs (magnification, x100). (H) Nestin-positive cells of passage culture of cortical NSCs (magnification, x200). NSC, neural stem cell.
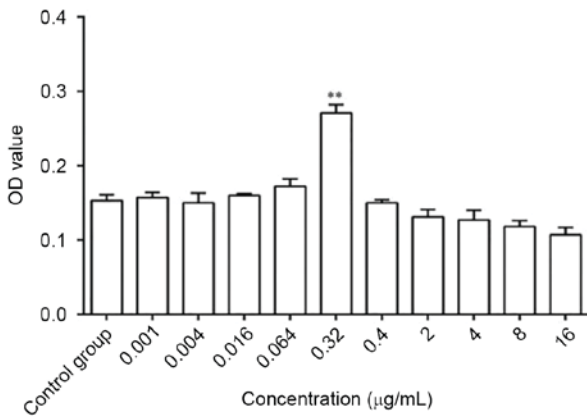

Figure 2. Optimum dose of ginsenoside-Rg1 for the proliferation of neural stem cells cultured for 3 days. **$P<0.01$ vs. the control group. OD, optical density.
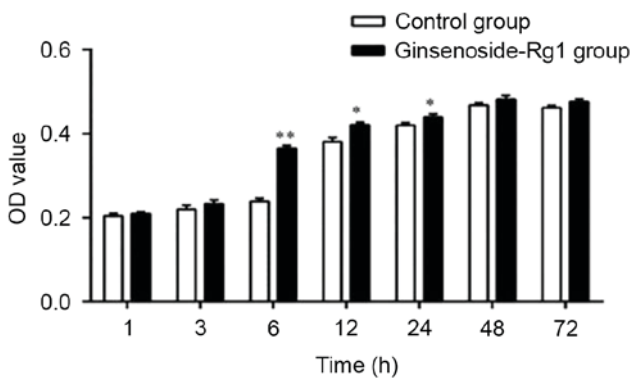

Figure 3. Effect of administration of 0.32 µg/ml ginsenoside-Rg1 at different time points on the proliferation of neural stem cells. *$P<0.05$, **$P<0.01$ vs. the control group. OD, optical density.

certain cells were binucleated. When nestin and vimentin were co-expressed in cultured cells, sizes of double-stained cells were different and cell bodies with processes were round or oval (Fig. 8). All positive cells visibly decreased in number in the OGD group and increased in the ginsenoside-Rg1

treatment group. Statistical analysis revealed that, compared with the OGD group, the area density, optical density, and the number of nestin and vimentin positive cells and nestin/vimentin double staining cells, increased significantly in the ginsenoside-Rg1 group (all $P<0.05$; Fig. 9).

Discussion

In previous studies NSCs have been successfully isolated from the striatum, hippocampus and cortex of the rat embryo (7-10). These cells have been demonstrated to proliferate and differentiate into neurons and glial cells, when stimulated with neurotrophic factors including the bFGF or epidermal growth factor (32,33). NSCs are normally cultured in serum-free culture medium. The main purpose of using serum-free medium is to remove confounding factors, including growth factors, which may induce the differentiation of NSCs. NSC culture medium is sometimes enriched with growth factor bFGF, as it promotes the survival and self-renewal of NSCs (34). In the present study, growth factor bFGF was added to the culture medium used for the early isolation of NSCs, and the primary and passaged culture of cortical NSCs; however, a serum-free medium was used during experiments using ginsenoside-Rg1 and OGD.

Nestin is an intermediate filament protein that is commonly used as a neural stem/progenitor cell marker and it can be defined as a class VI intermediate filament protein (35). Nestin expression begins during neurulation, decreases when the migration of nerve cells is completed and stops with the completion of the differentiation of NSCs (36,37). Therefore, nestin is a specific marker protein for neural stem cells. Embryonic rat cortical cells that were isolated and cultured in the present study expressed nestin. Sizes and morphology of nestin-positive cells were different, certain nuclei were offset, or were in possession of one or no cellular process, which suggested that NSCs were in an undifferentiated state. Therefore, the isolated cells in the present study exhibited the biological properties of NSCs. Nestin-positive NSCs increased in size and number following treatment with ginsenoside-Rg1. These results indicated that
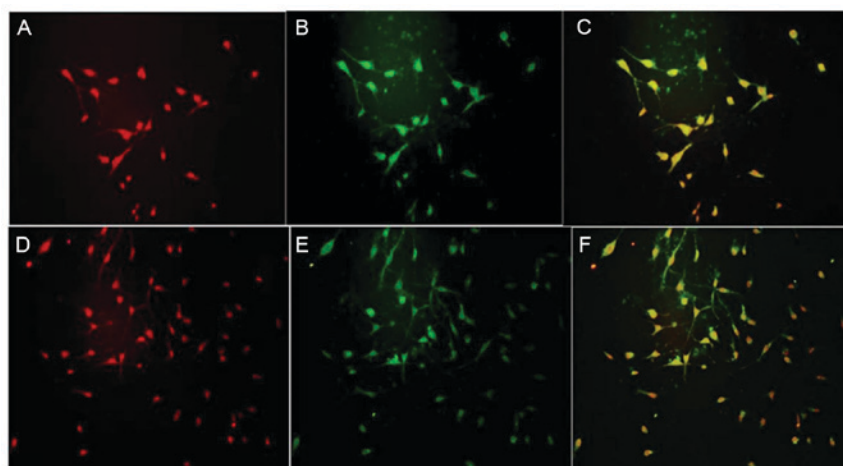
Figure 4. Effects of treatment with ginsenoside-Rg1 and oxygen glucose deprivation on neural stem cell proliferation and nestin/BrdU double staining, respectively (magnification, x100). The oxygen glucose deprivation group: (A) Nestin positive cells. (B) BrdU positive cells. (C) Merged image of nestin-positive and BrdU-positive cells. Ginsenoside-Rg1 group: (D) Nestin positive cells. (E) BrdU positive cells. (F) Merged image of nestin-positive and BrdU-positive cells. BrdU, bromo-2-deoxyuridine.
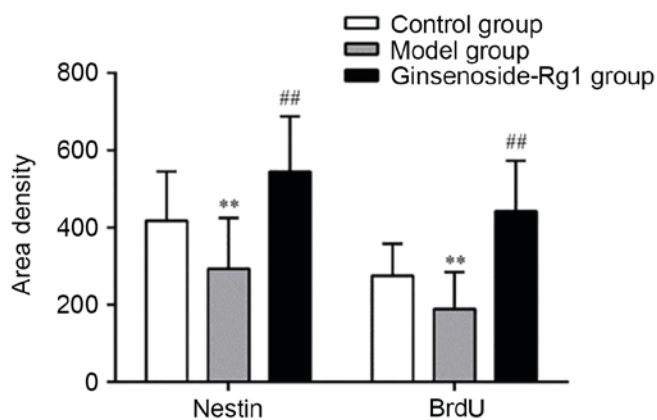

Figure 5. Area densities of nestin/Brdu double staining. **P<0.01 vs. the control group; ##P<0.01 vs. the model group. BrdU, bromo-2-deoxyuridine.

Figure 7. Number of nestin and BrdU-positive cells. **P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the model group. BrdU, bromo-2-deoxyuridine.
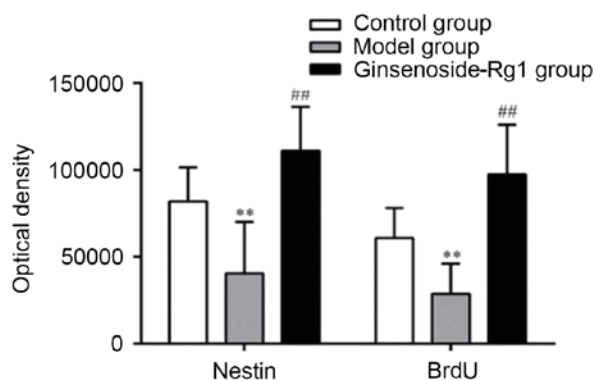

Figure 6. Optical densities of nestin/Brdu double staining. **P<0.01 vs. the control group; ##P<0.01 vs. the model group. BrdU, bromo-2-deoxyuridine.

ginsenoside-Rg1 had effects on the survival, proliferation and self-renewal of cortical NSCs of embryonic rats.

Ginsenoside-Rg1 promoted the proliferation of cortical NSCs of embryonic rats at a concentration of 0.32 μg/ml. Increasing the concentration of ginsenoside-Rg1 above this thresho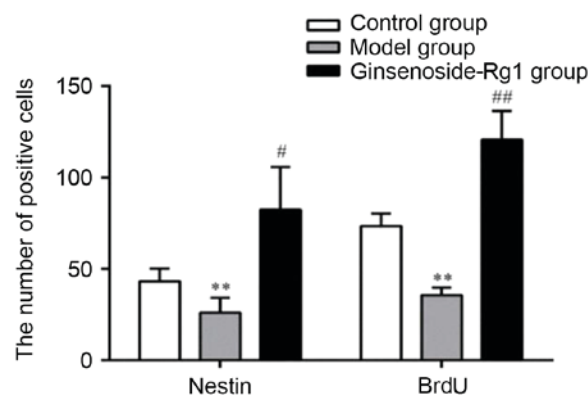ld gradually decreased the NSC proliferative effect. The OD values of cells treated with ginsenoside-Rg1 decreased compared with the control group when the ginsenoside-Rg1 concentration reached 2 μg/ml. Therefore, 0.32 μg/ml was the optimum dose of ginsenoside-Rg1 for the proliferation of cortical NSCs of embryonic rats. During the screening for the time window of ginsenoside-Rg1 administration at 0.32 μg ml^{-1} concentration, there was no difference in OD values between the ginsenoside-Rg1 and control groups when the chemical was added for 1-3 h. The efficacy of ginsenoside-Rg1-induced cell proliferation was higher after 6 h of treatment. Therefore 6 h was the optimum duration of incubation with ginsenoside-Rg1 at 0.32 μg ml^{-1} for the proliferation of NSCs.

BrdU is an analogue of thymidine, which can be incorporated into the DNA of differentiated cells in the S phase of the cell cycle (38). BrdU antibody does not cross-react with thymine, therefore BrdU incorporation can be observed by immunohistochemical staining in the cell, so that <1% being synthetic BrdU cells can be reliably detected (39). BrdU is a specific marker for proliferation of NSCs. BrdU and nestin/BrdU double-labeled positive cells increased in number
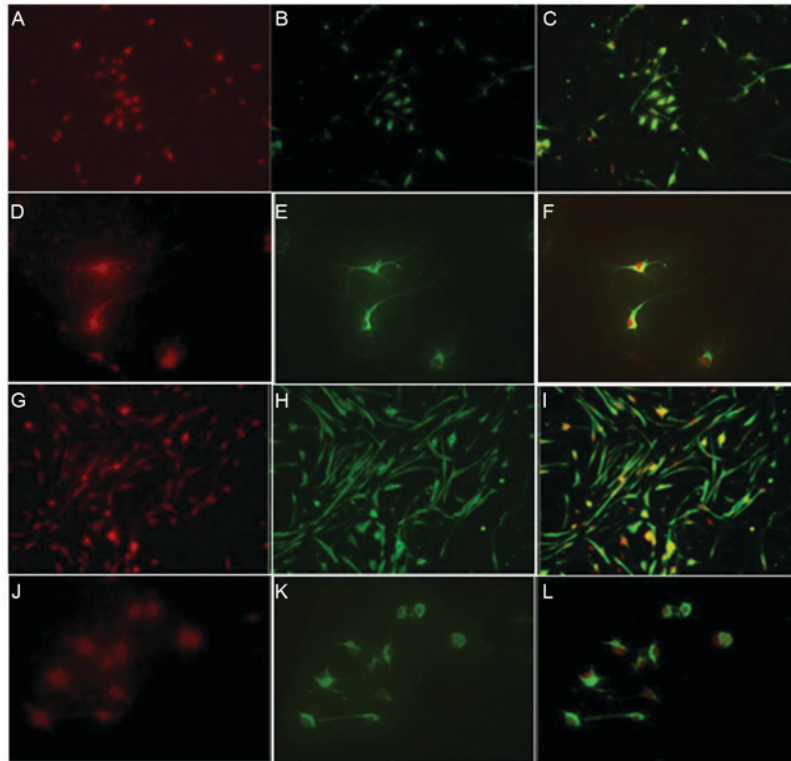
Figure 8. Effects of ginsenoside-Rg1 and oxygen glucose deprivation on the glial-like differentiation of cortical neural stem cells using nestin/vimentin double staining. OGD group (magnification, x100): (A) Nestin-positive cells. (B) Vimentin positive cells. (C) Merged image of nestin- and vimentin-positive cells (magnification, x100); OGD group (magnification, x200): (D) Nestin-positive cells. (E) Vimentin-positive cells. (F) Merged image of nestin- and vimentin-positive cells. Ginsenoside-Rg1 group (magnification, x100): (G) Nestin-positive cells. (H) Vimentin-positive cells. (I) Merged image of nestin- and vimentin-positive cells. Ginsenoside-Rg1 group (magnification, x200): (J) Nestin-positive cells. (K) Vimentin-positive cells. (L) Merged image of nestin- and vimentin-positive cells. OGD, oxygen glucose deprivation.

following treatment with ginsenoside-Rg1. Compared with the control group, there was a difference in the number, area and optical density of BrdU and nestin/BrdU double-positive cells in the ginsenoside-Rg1 group. These results indicated that ginsenoside-Rg1 had a significant, positive effect on the proliferation of NSCs.

NSCs are pluripotent and can differentiate into neurons and glial cells. Ginsenoside-Rg1 promoted the proliferation of NSCs and differentiation into vimentin-positive glial cells. The number of vimentin-positive cells in the ginsenoside-Rg1 group was increased compared with the control group, and certain cells were woven together or gathered into a group. The cell sizes also differed between vimentin-positive cells suggesting that they might not have been mature NSCs. Nestin/vimentin double staining was frequently observed. Since vimentin is a marker for naive glial cells and nestin is a marker of neural stem cells, it was concluded that ginsenoside-Rg1 promoted the differentiation of NSCs into glial-like cells.

The proliferation and differentiation of NSCs requires the presence of neurotrophic factors, and different growth factors affect NSC differentiation. As secretory cells, adult NSCs can promote certain growth factors to maintain neurogenesis in a paracrine and autocrine manner. Ginsenoside-Rg1 served a role in promoting the proliferation and glial-like-directed differentiation of cortical NSCs. A plausible explanation for these observations is that ginsenoside-Rg1 exhibits effects similar to growth factors, stimulating NSCs in an autocrine
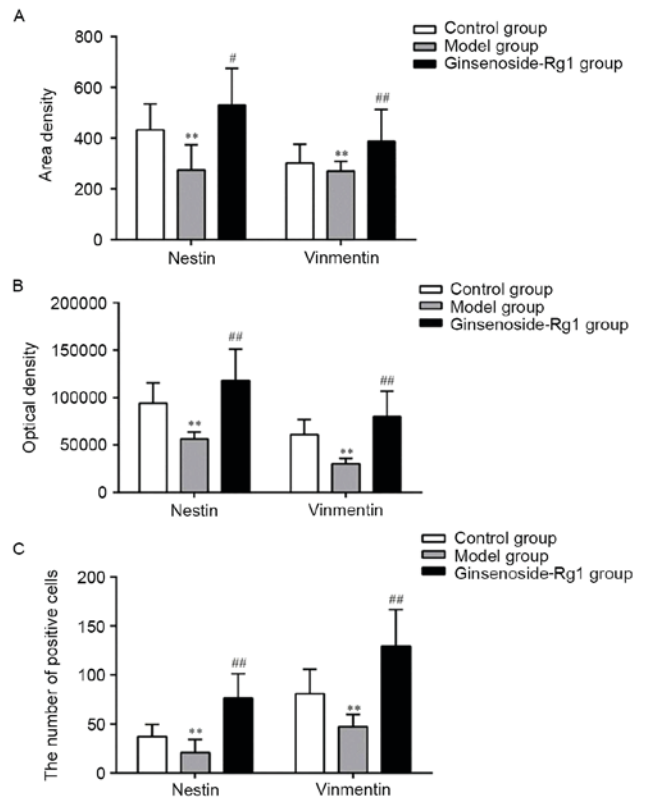

Figure 9. (A) Area density, (B) optical density and (C) number of nestin/vimentin-positive cells. $^{**}P<0.01$ vs. the control group; $^{#}P<0.05$, $^{##}P<0.01$ vs. the model group.

or paracrine manner to promote proliferation and differentiation of NSCs; however, the mechanism underlying these observations remain to be elucidated.

In conclusion, ginsenoside-Rg1 could maintain the survival and self-replication of embryonic rat cortical NSCs, and ginsenoside-Rg1 exhibited significant effects on proliferation and glial-like-directed differentiation. The mechanism of the proliferation and differentiation of NSCs remains unknown and can be studied further in the future.

Acknowledgements

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