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Original Article

Effects of *Panax notoginseng* saponins on proliferation and differentiation of rat embryonic cortical neural stem cells

Yinchu Si [a], Jun Zhu [b], Xiang Huang [c], Peichun Zhu [a], Chune Xie [d,*]

[a] *Department of Anatomy, Beijing University of Chinese Medicine, Beijing, China*
[b] *Department of Laboratory Medicine, University of California, San Francisco, CA, USA*
[c] *Scientific Experimental Center of Preclinical Medical School, Beijing University of Chinese Medicine, Beijing, China*
[d] *Dongfang Hospital, Beijing University of Chinese Medicine, Beijing, China*

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Abstract

Background: We aimed to study the effect of *Panax notoginseng* saponins (PNS) on the proliferation, differentiation, self-renewal, and expressions of basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (BDNF) in rat embryonic neural stem cells (NSCs).
Methods: Cortical stem cells were isolated from rat embryos on Embryonic Day 17 (E17) and identified by nestin expression. Subsequently, primary culture, subculturing, and single cell cloning were performed on the cells. After the first cell passage (P1), the cells were resuspended and divided into a control group and a treatment group. Control cells were cultured in serum-free basal culture medium with B27 and dulbecco's modified eagle medium (DMEM)/F12. The same medium supplemented with PNS (100 μg/mL) was used to culture cells in the treatment group. Both groups were incubated at 37°C in a 5% CO_2 incubator. Immunocytochemistry was performed 4 days after incubation.
Results: Primary, P1, and P2 cells in the treatment group formed neurospheres, as did single cell clones of the P1 cells in this group. After being cultured for 4 days, the number of nestin-, proliferating cell nuclear antigen (PCNA)-, Tuj-1-, neurofilament (NF)-, vimentin-, glial fibrillary acidic protein (GFAP)-, bFGF-, and BDNF-positive cells significantly increased in the treatment group in comparison to the control group. All positively stained cells could form clear clusters.
Conclusion: PNS can promote rat embryonic cortical NSC survival, self-renewal, proliferation, and differentiation through neurotrophic factors by autocrine or paracrine signaling.

Keywords: basic fibroblast growth factor; brain-derived neurotrophic factor; cell culture; embryonic rat; neural stem cells; *Panax notoginseng* saponins

1. Introduction

Neural stem cells (NSCs) were initially described in the early 1990s. They are self-renewing, multipotent cells that can differentiate into neurons, astrocytes, and oligodendrocytes in the nervous system.[1–5] This discovery contradicted the general belief that neuronal cells are not capable of regeneration, thus creating hope for patients with nervous system injuries and neurodegenerative diseases. Since then, there has been an increase in research related to the biological and pharmaceutical characteristics of NSCs. Therapies using NSC technology have emerged as a viable alternative in the treatment of neural injuries.[6–10] Traditional Chinese medicine (TCM) comprises a broad set of medical practices developed in China over 5000 years, providing health services not only to the Chinese people, but also worldwide. TCM has previously demonstrated efficacy in the prevention and treatment of

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Correspondence to: Dr. Chune Xie, Dongfang Hospital, Beijing University of Chinese Medicine, 6, District 1 Fangxingyuan, Fangzhuang Fengtai District, Beijing 100078, China.
E-mail address: chunexie@126.com (C. Xie).

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diseases of the central nervous system.[11,12] Thus, the effect of TCM on NSCs has become a major topic of research in the Chinese neuroscience community, and an integral part of medical research worldwide.

NSCs are found in many encephalic regions of the rat embryo. These cells proliferate vigorously and eventually differentiate into neurons and glia. NSCs that persist in the adult rat brain are found mainly in the ventricular subependymal and subgranular zone, where they are relatively inactive or proliferate slowly. In previous studies, NSCs have been successfully isolated from the striatum, hippocampus, and cortex of the rat embryo. These cells have been shown to proliferate and differentiate into neurons and glial cells under the influence of neurotrophic factors, such as the basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF).[13–16] Therefore, embryonic rats NSCs are suitable for *in vitro* experiments.

Panax notoginseng saponins (PNS) is the effective active ingredient of Sanqui of Chinese herbs. The study reported that PNS has neuroprotective effects after stroke (cerebral hemorrhage and cerebral ischemia) through anti free radicals,[17] reducing the apoptosis of nerve cells and neurotoxicity,[18,19] and promoting angiogenesis and the synthesis and release of neurotrophic factors. Recently, it has been reported that PNS can promote the proliferation and differentiation of cells in the subependymal zone of the lateral ventricle after rat cortical devascularization, and it also promotes the expression of bFGF.[20]

In the present study, NSCs from embryos at Embryonic Day 17 (E17) were used to investigate the influence of PNS on their proliferation and differentiation. Cell culture and immunocytochemical techniques were used to investigate the mechanism of the bioactive effects of PNS.

2. Methods

2.1. Experimental animals

E17 Wistar rats were provided by the Animal Institute, Beijing, China. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Beijing University of Chinese Medicine, China.

2.2. Primary culture

Under sterile conditions, the cortical tissue of the E17 Wistar rats was isolated, and the meninges peeled off. The cortexes were then cut into small pieces (1 mm) in dulbecco's modified eagle medium (DMEM)/F12 (1:1) after removing any remaining blood with a dissecting solution (D-Hanks, composed of KCl, KH_2PO_4, NaCl, $NaHCO_3$, and Na_2HPO_4*$12H_2O$). These pieces were digested for 25 minutes at 37°C by adding 0.125% trypsin solution, and were agitated twice during digestion. The digestion was then terminated by adding 10% fetal calf serum, and the tissue was washed with DMEM/F12 medium to remove the serum. A single cell suspension was achieved by repeatedly blowing and stirring using a pipette. The number of viable cells in the suspension was assessed using trypan blue, and cell density of the suspension was adjusted to 2×10^5 cells/mL with serum-free DMEM/F12 culture medium supplemented with B27. This single cell suspension was then seeded into 25-mL culture flasks coated with polylysine (5 mL/flask).

2.3. Cell passaging, single cell cloning, and grouping

Cell passaging and single cell cloning were carried out using primary rat cells after 7 days of incubation.

Primary cells were passaged to a density of 2×10^4 cells/mL. After 7 days, these P1 cells were repassaged to a density of 2×10^4 cells/mL and then labeled P2 cells. Single cell cloning was performed simultaneously during the passaging of primary cells. First, cultured primary cells were diluted with culture medium to a density of 1–2 viable cells/mL, as determined by cell counting, and seeded into 96-well culture plates (100 μL medium/well). Observations and results were recorded from wells that only contained one cell. The grouping and incubation conditions adopted for the P1, P2, and the single-cloned cells were the same as those for the primary cells, except that the culture flasks and plates were not coated with polylysine, which allows the cells to grow in a suspension.

During the experiments, the single cell suspensions of P1 cells were divided into a control group and a treatment group. Control cells were cultured in serum-free DMEM/F12 medium supplemented with B27, while the treatment culture was supplemented with 100 μg/mL PNS. They were both incubated at 37°C in a 5% CO_2 incubator.

2.4. Screening of the optimal dose of PNS on the cortical NSC by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method

PNS (lot number: 07032-9812, purity > 98%) was provided by the Kunming Pharmaceutical Corporation, Kunming, China.

NSCs were collected by centrifugation, single cell suspension was prepared by mechanical pipetting and separating, and NSCs were seeded with 1×10^5/mL of cell density in 96-well culture plates, 100 μL/hole, cultured with serum-free medium. Groups including the control group and PNS groups (gradient concentrations include 0.77 μg/mL, 1.54 μg/mL, 3.85 μg/mL, 7.7 μg/mL, 15.4 μg/mL, 30.8 μg/mL, 61.6 μg/mL, 123.2 μg/mL, 246.4 μg/mL, and 492.8 μg/mL), with eight holes for a group, were cultured for 3 days. We added 20 μL of methyltrichlorosilane (MTS)/PMS solution/hole for 3 hours before termination of culture, then continuously cultured at 37°C for 3 hours, and the optic density (OD) value was detected at a wavelength of 492 nm.

2.5. Screening of the best window time of the best dose of PNS on the cortical NSC by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method

Based on the screening of the best dose of PNS above, NSCs were collected by centrifugation, single cell suspension was prepared by mechanical pipetting and separating, NSCs were seeded with 1×10^5/mL of cell density in 96-well culture plates, 100 μL/hole, and cultured with serum-free medium. Time points included 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours. Every time point was divided into the control group and the treatment group. NSCs were cultured for 3 days, adding 20 μL of methyltrichlorosilane (MTS)/PMS solution/hole at 3 hours before termination of culture, and then continuously cultured at 37°C for 3 hours, and the OD value was detected at a wavelength of 492 nm.

2.6. Immunocytochemistry

After 4 days of incubation under the described conditions, immunocytochemistry was performed in the P1 control and treatment groups in order to analyze cell proliferation, differentiation, and expression of nestin, bFGF, and brain-derived neurotrophic factor (BDNF). The specific antibodies used in our experiments were as follows: antibody against the protein nestin, to label NSCs or NSC precursors; anti-proliferating cell nuclear antigen (PCNA), to detect the proliferation of NSCs or their precursors; anti-Tuj-1 and anti-neurofilament (NF) antibodies, to detect immature and mature neurons; anti-vimentin and anti-glial fibrillary acidic protein (GFAP), to detect immature and mature glial cells. We also analyzed bFGF and BDNF expression.

The avidin-biotin complex (ABC) method was used in the immunocytochemical procedures. First, culture plates were washed with 0.01M phosphate-buffered saline (PBS; pH 7.2) three times after removing the culture medium with a pipette. The P1 cells on the plates were then: (1) fixed with 4% paraformaldehyde/0.1M PBS (pH 7.2–7.4) for 20 minutes, and then washed three times with 0.01M PBS (5 minutes each time); (2) incubated in 1% 0.01M PBS three times (5 minutes each time); (3) blocked with 10% normal goat serum and incubated at room temperature for 20 minutes; (4) incubated with the primary antibody at 4°C overnight; (5) incubated with streptavidin horseradish peroxidase (S/HRP) working solution (Zymed, USA) at 37°C for 2 hours, and then washed with 0.01M PBS three times (5 minutes for each time); (6) stained with 0.05% 3,3'-diaminobenzidine-0.01% H_2O_2 solution; and (7) dehydrated and mounted for microscopy.

No primary antibody was used in the immunocytochemical staining of P1 cells in the negative control; 0.01M PBS was used instead. Primary antibodies used in the experiments were mouse anti-nestin (1:500, Pharmingen, USA), mouse anti-PCNA (1:80, Zymed, USA), rabbit anti-Tuj-1 (1:1000, R&D, USA), mouse anti-NF (1:80, Santa Cruz, USA), mouse anti-vimentin (working solution, Sigma, USA), rabbit anti-GFAP (1:500, Zymed, USA), rabbit anti-bFGF (1:800, Santa Cruz, USA), and rabbit anti-BDNF (1:80, Santa Cruz, USA). All of the primary antibodies were diluted in 0.01M PBS.

2.7. Image treatment and statistical analysis

The numbers of nestin-, PCNA-, Tuj-1-, NF-, vimentin-, and GFAP-positive cells were measured and analyzed using the C8CMIAS True Color Image Analysis System (Beijing University of Aeronautics and Astronautics, Beijing, China). In the image analysis, three slices were randomly chosen from each group (control and treated), and three visual fields were randomly analyzed from each slice. The number of positive cells in each field was recorded; the mean of positive cells (mean ± standard deviation) was used to denote the number of positive cells in each group. The *t* test was used to compare results between groups.

3. Results

3.1. Effects of PNS on the primary culture and single cell cloning of P1 cells of rat embryonic cortical cells

Phase contrast microscopy revealed that primary culture rat embryonic cortical cells in both the control and treatment groups attached to the surface of the culture flask 2 hours after being plated. Some cells had two to three nucleoli and one to two short, thin processes. After 3–4 days in culture, the number of cell processes increased. We found that there were more cells in the treatment group than in the control group. Three to four days after plating, the cells in the treatment group were larger and had more processes than in the control group. We also noticed that the treatment group had some clear floating neurospheres of different sizes, composed of tens or hundreds of cells. Cells in the center of the neurospheres had no processes, were translucid and rather small in size, and contained one to two nucleoli. Cells at the periphery had one to two processes. Similar neurospheres were observed in the treatment group of the P1 and P2 cells after 3–4 days of incubation. The control group had fewer neurospheres and smaller cells (Fig. 1A and 1B).

During the single cell cloning of P1 cells, observation of the 96-well culture plates revealed that after 3–4 days of incubation, two to three single cells in the treatment group formed a neurosphere, composed of tens or hundreds of cells. The sizes of the neurospheres were different depending on the different single cells that formed them. No neurospheres were observed in the control group 3–4 days after plating (Fig. 1C and 1D).

3.2. A 15.4 μg/ml dose of PNS is the best dose on the cortical NSCs cultured for 3 days

The OD value clearly increased at 0.77–123.2 μg/mL of PNS measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), and when compared with the control group they were significant ($p < 0.05$ or $p < 0.01$), wherein the highest OD value was detected at a dose of

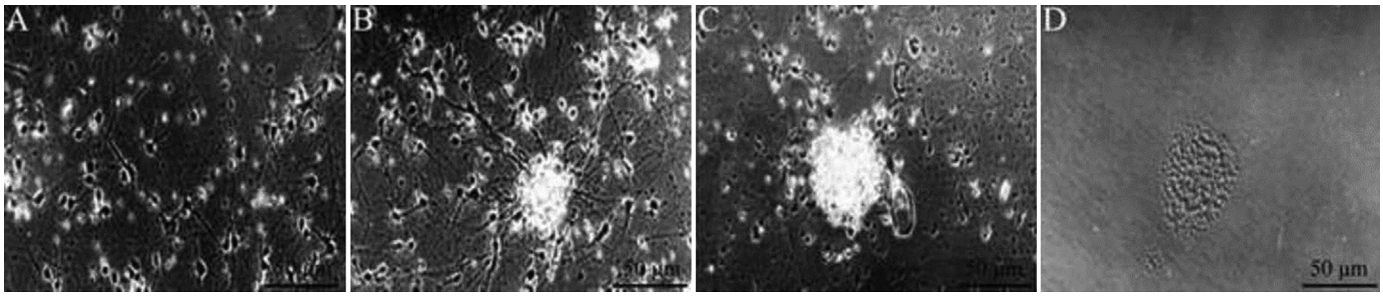

Fig. 1. Embryonic cortical stem cells culture (200×). (A) the primary cell of control group cultured for 4 days which did not form neurosphere; (B) the primary cell of treatment group cultured for 4 days forming neurosphere; (C) the P1 cell of treatment group cultured for 4 days forming neurosphere; (D) neurosphere formed from monoclone in the treatment group. ABC method ×200. ABC = avidin-biotin complex.

15.4 μg/mL (compared with control group, $p < 0.01$); regarding the OD value of the 246.4 μg/mL group compared with the control group, there was no significant difference ($p > 0.05$); therefore, the dose of 15.4 μg/mL was used in the next experiments.

3.3. The time point of 15.4 μg/mL of PNS on the cortical NSCs is 6 hours

OD values of NSCs treated with 15. 4 μg/mL of PNS after 1–3 hours compared with control groups had no significant difference ($p > 0.05$); OD values of NSCs increased greatly at 6 hours, compared with the control group, resulting in significant differences ($p < 0.01$); therefore, 6 hours was used in the Oxygen and glucose deprivation (OGD) experiment.

3.4. Effect of PNS on nestin and PCNA expression

Nestin expression could be detected in P1 rat embryonic cortical cells after 4 days of cell culture in both the control group (Fig. 2A) and treatment group (Fig. 2B). Nestin-positive cells varied in size and the presence or absence of apophyses. Positive dark brown staining was observed in the cytoplasm. These stained cells were round or oval-shaped; their nuclear zones were not stained and often displaced to one side of the cell. Most nestin-positive cells had a single process, and their chromosomes could be clearly seen under a high power lens. In the treatment group, nestin-positive cells were often clustered, with three kinds of cells (neuron, astrocyte, and oligodendrocyte) found within the clusters. They varied in size, were round in shape, and had no apophyses, while most nestin-positive cells at the periphery of the clusters had apophyses. The number of nestin-positive cells in the control group was significantly lower than that in the treatment group. In addition, the cells were smaller and did not form clear clusters.

PCNA expression was confirmed in the nucleus owing to the presence of a dark brown color after being stained. The nucleoli of the PCNA-positive cells were round, oval-, or rod-shaped, but mostly either round or oval. The size of the nucleoli varied in the PCNA-positive cells, and sometimes two fused nucleoli could be found. Compared with the control (Fig. 2C), the treatment group contained more PCNA-positive cells, and had larger cells with more nucleoli. Staining was also more prominent in the treatment group (Fig. 2D).

Cell counting showed that the average number of nestin- and PCNA-positive cells was greater in the treatment group compared to the control group. Statistical analysis showed that there was a significant difference between the results of the two experimental groups ($p < 0.01$, Table 1).

3.5. Effects of PNS on Tuj-1 and NF expression

A dark brown color in the cytoplasm confirmed the expression of Tuj-1. The nuclear area of a Tuj-1-positive cell

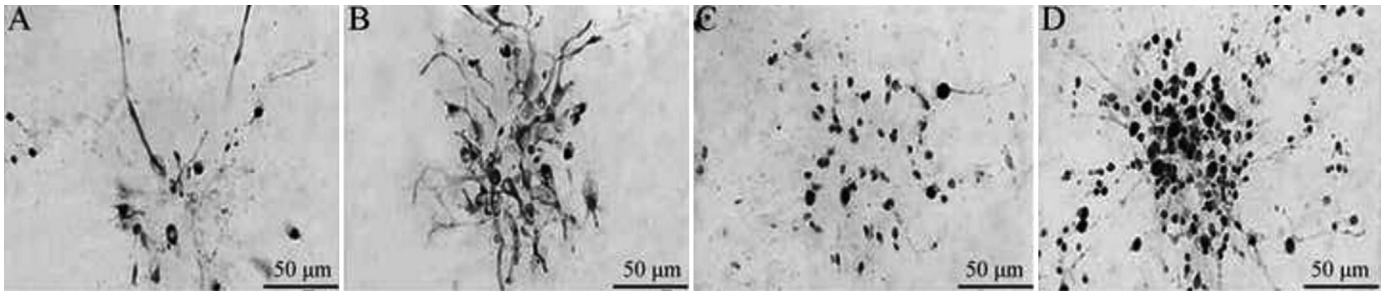

Fig. 2. Nestin and PCNA expressed in P1 rat embryonic cortical cells (200×). (A) Nestin-positive cell of cortical neural stem cell from embryonic rat brain of control group; (B) nestin-positive cell of cortical neural stem cell from embryonic rat brain of treatment group; (C) PCNA-positive cell of cortical neural stem cell from embryonic rat brain of control group; (D) PCNA-positive cell of cortical neural stem cell from embryonic rat brain of treatment group. ABC method ×300. ABC = avidin-biotin complex; Nestin = neuroepithelial stem protein for marker of neural stem cell; PCNA = proliferating cell nuclear antigen for marker of proliferation.

Table 1
Mean number of immunopositive cells of control and treatment cortical stem cells of embryonic rats in vitro (n = 9).

Group	Nestin	PCNA	Tuj-1	NF	Vimentin	GFAP
Control group	16.89 ± 10.12	14.22 ± 4.55	67.78 ± 9.58	19.22 ± 6.67	27.44 ± 4.72	49.67 ± 8.75
Treatment group	43.22 ± 7.41**	30.33 ± 10.67**	103.22 ± 8.91**	42.89 ± 5.90**	72.56 ± 9.55**	101.4 ± 11.81**

Data are expressed as the mean ± standard deviation.
**$p < 0.01$, different from control.
GFAP = glial fibrillary acidic protein; NF = neurofilament; PCNA = proliferating cell nuclear antigen.

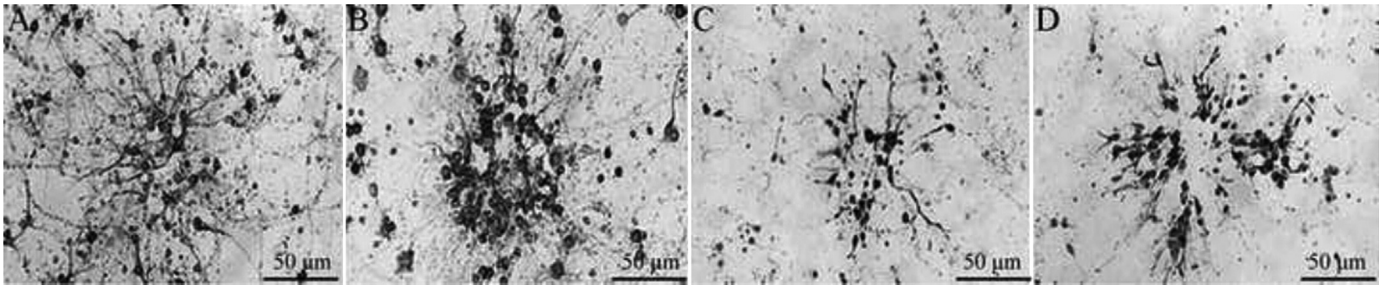

Fig. 3. Tuj-1 and vimentin expressed in P1 rat embryonic cortical cells (200×). (A) Tuj-1 positive cell of cortical stem cell from embryonic rat brain of control group; (B) Tuj-1 positive cell of cortical stem cell from embryonic rat brain of treatment group; (C) vimentin-positive cell of cortical stem cell from embryonic rat brain of control group; (D) vimentin-positive cell of cortical stem cell from embryonic rat brain of treatment group. ABC method ×300. ABC = avidin-biotin complex; Tuj-1 = Class III P-tubulin for marker of neuronal precursor; Vimentin = a marker of glial precursor.

was not stained and often displaced to one side of the cell. Under a high power lens, the nucleolus of the cell was visible. In the treatment group (Fig. 3B), some Tuj-1-positive cells were small, round, and had no apophyses, while others were large, round or oval-shaped, and had one or more apophyses. In addition, Tuj-1-positive cells were gathered into clusters. In the control group (Fig. 3A), fewer Tuj-1-positive cells were found; they were smaller and did not form clear clusters.

In the NF-positive cells, the dark brown NF staining was localized to the cytoplasm. In the treatment group, NF-positive cells were round or oval-shaped and had one or more apophyses. In the control group, there were fewer NF-positive cells, and most of them were small, round, and had no apophyses.

Cell counting showed that the average number of Tuj-1-positive and NF-positive cells in the treatment group was greater than that in the control group. Statistical analysis of the experimental results showed that there was a significant difference ($p < 0.01$) between the results of the two groups in the experiment (Table 1).

3.6. Effects of PNS on vimentin and GFAP expression

A dark brown stain confirmed vimentin expression in the cytoplasm of rat embryonic cortical cells. The nuclear area of a vimentin-positive cell was negative and displaced to one side of the cell. In the treatment group (Fig. 3D), there were three kinds of vimentin-positive cells. They were round or oval-shaped, and had one or more apophyses. The cells formed clusters; they gradually became larger from the center to the periphery, and the number of apophyses increased. In the control group (Fig. 3C), fewer vimentin-positive cells were found, and they did not form clusters. In addition, most cells were small, round, and had one or more apophyses.

Expression of GFAP was observed in the cytoplasm as a dark brown color. In the treatment group, some GFAP-positive cells were large and triangular-shaped with more than one process, while others were small, round or oval-shaped, and had one or more apophyses. In the control group, GFAP-positive cells were small and round; most of the cells had one process, while some had more than one.

The average number of vimentin-positive and GFAP-positive cells in the treatment group was greater than that in the control group, as determined by cell counting. Statistical analysis of the results showed that there was a significant difference ($p < 0.01$) between the results of the two groups (Table 1).

3.7. Effect of PNS on bFGF and BDNF expression

bFGF and BDNF expressions were observed in rat embryonic cortical cells (P1) after 4 days of culture in cells grown both in the presence (treatment group, Fig. 4B and 4D) and absence of PNS (control group, Fig. 4A and 4C). Staining was localized to the cytoplasm. Positively stained cells were round or oval-shaped; some of them had one or two apophyses. In the treatment group, the bFGF- and BDNF-positive cells were clustered, and the cells with apophyses were usually located in the periphery. The treatment group also had more bFGF- and BDNF-positive cells than the control group, and the staining was more intense. Moreover, in the control group, bFGF- and BDNF-positive cells were usually observed in the dispersed state, not in clear clusters.

4. Discussion

NSCs can potentially differentiate into many cell types, such as neurons, astrocytes, and oligodendrocytes. They have

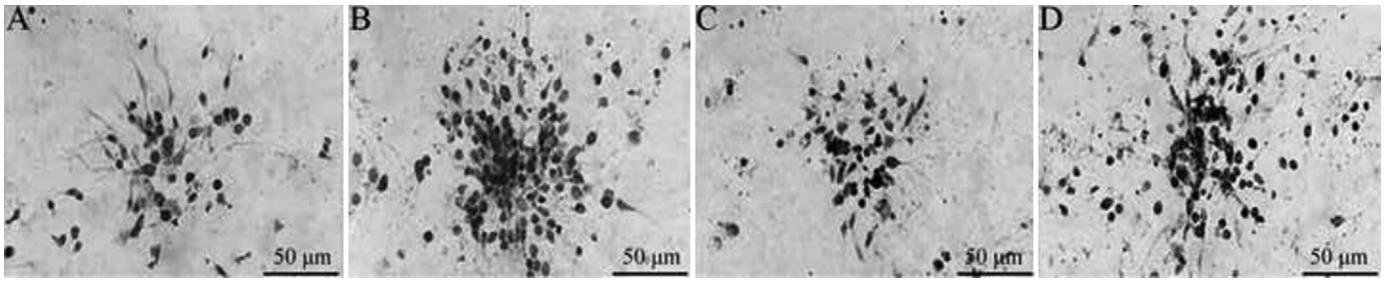

Fig. 4. Basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (BDNF) expressed in P1 rat embryonic cortical cells (200×). (A) bFGF-positive cell of cortical stem cell from embryonic rat brain of control group; (B) bFGF-positive cell of cortical stem cell from embryonic rat brain of treatment group; (C) BDNF-positive cell of cortical stem cell from embryonic rat brain of control group; (D) BDNF-positive cell of cortical stem cell from embryonic rat brain of treatment group. ABC method ×300. ABC = avidin-biotin complex.

the capacity to self-renew and produce many neuronal cells.[2,5] The results from our present study show that PNS can promote the survival, proliferation, and self-renewal of rat embryonic cortical NSCs, as well as promote their differentiation into neurons and glial cells.

Nestin, a type of intermediate filament protein, is a specific marker for embryonic NSCs.[21] NSCs had previously been successfully isolated from the striatum, hippocampus, and cortex of rat embryos. Previous in vitro studies have proved that despite their different locations within the central nervous system, NSCs are characterized by their expression of nestin, their capacity for continuous proliferation, self-renewal, and multidirectional differentiation under the presence of bFGF or both bFGF and EGF, and their capacity for differentiation into neurons and glia under other specific conditions.[18–27] Rat embryonic cortical cells isolated and incubated in the present study expressed nestin, which identifies them as NSCs. Our results showed that PNS can increase the number of nestin-expressing NSCs or NSC precursors, suggesting that PNS might promote the survival of these cells, as well as their proliferation and self-renewal by inducing the production of more undifferentiated, nestin-positive cells that possess the characteristics of NSC precursors. It was also observed that under the influence of PNS, PCNA expression increased, indicating that PNS can indeed promote the proliferation of rat embryonic cortical cells. PCNA is associated with DNA duplication and repair; it is a cofactor necessary for the function of DNA polymerase 5 and 8. In the present study, PCNA was used to identify cells in the process of proliferation and division.[28,29] Nestin-positive cells cultured in the presence of PNS varied in shape and size, suggesting that these cells may be in different phases of differentiation. Moreover, the nucleoli of some nestin-positive cells were displaced to one side of the cell and had one or no processes, suggesting that they may be in a primordial stage. An earlier study reported that PNS can promote the proliferation of rat hippocampal NSCs *in vitro*[21] and expression of nestin, PCNA, and bFGF in the subependymal cells of the lateral ventricle after rat cortical devascularization *in vivo*. These reported results are in agreement with our results, indicating that PNS has effects on the proliferation of NSCs.[20]

NSCs can continuously divide into more undifferentiated cells possessing the same characteristics, thus demonstrating their capacity for self-renewal. This has been well proven in previous in vitro experiments. Investigators have found that cultured NSCs under the influence of bFGF or EGFs form clusters composed of many cells known as neurospheres. When cells in the neurospheres are singly cloned, they retain the ability to form neurospheres when cultured again in the presence of bFGF or EGF. At the same time, neurospheres can be induced to differentiate into neurons and glial cells (including astrocytes and oligodendroglial cells). If single cell clones in the neurospheres are further singly subcloned, they can again form neurospheres with a capacity for multidirectional differentiation. Such cloning processes can go for many generations, indicating that these cells also have the capacity for self-renewal and multidirectional differentiation. Thus, NSCs can also be called neurospheric cells. The existing studies show that neurospheres can easily form under suspension conditions, and all neurospheric cells can express nestin. We found in the present study that there were many neurospheres composed of primary, P1, or P2 cells in the treatment group. Under the influence of PNS, single cells cloned from P1 cells formed neurospheres and nestin-positive cell clusters, indicating that these neurospheres and clusters were formed by NSCs that possessed the ability for self-renewal. The various sizes of the clusters and neurospheres suggested that they were different in their primitive state and their ability to self-renew, and PNS can promote self-renewal and self-maintenance.

In the present study, we found that PNS could promote the differentiation of rat embryonic cortical NSCs into both Tuj-1- and NF-positive neurons, and vimentin- and GFAP-positive glial cells. All positive cells were found to be gathered into clusters, and the sizes of the cells in these clusters were different, suggesting that they may not be fully mature and could still be in different stages of differentiation. The cells at the center of these clusters were rather immature and had a round shape without any apophyses, while the cells at the periphery were highly differentiated, mature, larger, and often presented apophyses. The results of this experiment are consistent with the reported PNS promoting differentiation of rat hippocampal NSCs into neurons and astrocytes.[21]

Our results show that, compared with the control group, PNS promoted the expression of bFGF and BDNF in primary rat embryonic cortical cells. Neurotrophic factors play an important role in the regulation of proliferation and differentiation of NSCs; NSCs can survive and continuously proliferate only in the presence of neurotrophic factors, such as bFGF and EGF. Of these, bFGF has been proven to be an important mitogen, promoting the proliferation and division of NSCs.[30–32] Different neurotrophic factors have different inducing effects; for example, BDNF and NT-3 may promote NSC differentiation into neurons,[33,34] while ciliary neurotrophic factor and platelet-derived growth factor may promote NSC differentiation into astrocytes.[35] Several reports have confirmed these characteristics, but few have investigated the ability of NSCs to survive, proliferate, differentiate, and self-renew (promoted by the expression of neurotrophic factors). Our results showed that in the treatment group, bFGF- and BDNF-positive cells formed clusters, and bFGF and BDNF expressions were abundant, suggesting that PNS could promote the synthesis of bFGF and BDNF by rat embryonic cortical NSCs or NSC precursors. By this mechanism, rat embryonic cortical NSCs can not only promote their own survival, proliferation, differentiation, and self-renewal by autocrine secretion, but also can induce other NSCs by paracrine secretion. However, further studies are needed in order to better understand these mechanisms.[36]

Acknowledgments

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