Effects of passage and cryopreservation on neurotrophic factor secretion from choroid plexus epithelial cells

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Abstract. The aim of the present study was to evaluate the effects of passage and cryopreservation of choroid plexus epithelial cells on their secretion of neurotrophic factors. Choroid plexus epithelial cells were cryopreserved and thawed following primary culture or passage cultured for up to two passages. The supernatant of primary, first/second passage and cryopreserved-thawed choroid plexus epithelial cells was collected when cells reached 80-90% confluence. ELISA was used to quantify brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) levels in the cell supernatant. First passage and cryopreserved-thawed cells secreted less BDNF and CNTF compared with primary cultured cells and increased levels of these two factors compared with second passage cells, and increased levels of GDNF and NGF compared with primary cultured and second passage cells (all $P<0.05$). Therefore, first passage culture decreased BDNF and CNTF secretion but increased NGF and GDNF compared with primary culture; second passage culture diminished neurotrophic factor secretion compared with first passage culture; and cryopreservation did not weaken the function of choroid plexus epithelial cells in secreting BDNF, GDNF, NGF and CNTF. The current study demonstrates that first passage and cryopreserved-thawed choroid plexus epithelial cells have an enhanced function to secrete neurotrophic factors including BDNF, GDNF, NGF and CNTF.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FBS, fetal bovine serum; GDNF, glial cell-derived neurotrophic factor; NGF, nerve growth factor

Key words: choroid plexus epithelial cells, neurotrophic factors, passage culture, cryopreservation

Introduction

The choroid plexus, located within the brain ventricles, is a specialized structure consisting of epithelial cells and underlying vascular-rich connective tissues (1). Choroid plexus epithelial cells are involved in the production of cerebrospinal fluid, and secrete a number of neurotrophic factors including brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) (2-4). The neurotrophic factors are known to stimulate neuronal growth and promote neurite outgrowth (5). Depletion of these neurotrophic factors has been associated with pathologies and symptoms of Parkinson's, Alzheimer's and Huntington's diseases and spinal cord injury, and replacement strategies are considered as potential therapeutics for these neural degenerative diseases (3).

In recent years, cell transplantation therapy has emerged as a promising therapeutic option for neurorepair (6). Transplantation of choroid plexus epithelial cells from primary culture has been tested in several animal models, including rat models of Parkinson's disease, spinal cord injury and cerebral ischemia (7-9). However, cells are difficult to obtain in primary culture, and therefore the source of cells is limited. This problem may be overcome if passage culture and cryopreserved-thawed cells can be used for transplantation. This may depend on whether passage culture and cryopreservation impair the secretion of neurotrophic factors from choroid plexus epithelial cells.

To the best of our knowledge, there has been no investigation into the effects of passage culture and cryopreservation on neurotrophic factor secretion from choroid plexus epithelial cells. The present study was conducted to compare the levels of BDNF, GDNF, NGF and CNTF secreted by neonatal rat choroid plexus epithelial cells among primary, first passage and second passage cultures and cryopreserved-thawed cells.

Materials and methods

Animals and reagents. Neonatal male Sprague-Dawley rats (1-day-old, weighing 5-6 g) and their mothers were supplied by the Center of Experimental Animals, Xi'an Jiaotong University (Xi'an, China). All rat mothers were housed individually with their offspring in polypropylene cages in a standard animal room maintained at 22±3˚C and 50±20% humidity, and allowed access to food and water ad libitum under a natural day/night cycle. The experiments were performed with 24 rat offspring. The protocols for animal care and experimental management were approved by the Xi'an Jiaotong University Animal Experimentation Committee. Ethical approval for the study was obtained from the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University.

Dulbecco's modified Eagle's medium (DMEM, low glucose) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and 6-well plates were obtained from Corning Inc. (Corning, NY, USA). Recombinant rat epidermal growth factor (EGF) was obtained from PeproTech, Inc., (Rocky Hill, NJ, USA). ELISA kits for BDNF (cat. no. F15100), GDNF (cat. no. F15600), NGF (cat. no. F16310) and CNTF (cat. no. F15220) and normal goat serum were obtained from Shanghai Xitang Biological Technology Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) and trypan blue were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Primary culture. Primary culture of choroid plexus epithelial cells was prepared via the procedures described in our previous studies (10,11). Briefly, following euthanasia with an overdose of pentobarbital (150 mg/kg, intraperitoneal injection) and disinfecting with 75% ethanol, the rat brains were isolated and the choroid plexus was removed from the lateral ventricles. The tissues were segregated into small cell aggregates by a mechanical method, and cultured in DMEM supplemented with 10% FBS and 10 ng/ml EGF on 6-well plates at 37˚C in a humidified incubator at 5% CO_2. The culture medium was replaced every 2 days.

Passage culture and cell cryopreservation. When primary culture cells reached ~90% confluence, cells were detached with a 0.25% Trypsin-EDTA solution. Cell numbers were determined with a hemocytometer. The viability of cells was assessed by trypan blue exclusion test (12). Then, cells were divided into two equal groups: One group was used for cryopreservation, and the other group was seeded into 6-well plates at a density of $1.5x10^3$ cells/ml with DMEM containing 10% FBS, and incubated in a 37˚C, 5% CO_2 incubator. The culture medium was replaced as before every 2 days. When first passage culture cells reached ~90% confluence, the second passage was initiated and performed as above.

Cryopreservation and thawing of cells. Choroid plexus epithelial cells collected from the previous steps were aliquoted at a density of $2x10^6$ cells/cryovial with DMEM containing 90% FBS and 10% DMSO. Cryovials with primary cultures were incubated sequentially at 4˚C for 0.5 h, -20˚C for 2 h, and -80˚C for 16 h, and then transferred to and stored in a liquid nitrogen tank (-196˚C) until use. After 14 days of cryopreservation, the cells were thawed in a 37˚C water bath (~1 min). Upon thawing of the cells, the cryovial was removed from the water bath and the cells were transferred to a centrifuge tube containing 6 ml DMEM containing 10% FBS. Following centrifugation at 1,000 x g for 5 min at room temperature, cells were resuspended with 9 ml complete medium and dispensed into a 6-well plate at a density of $1.5x10^3$ cells/ml. Thereafter, culture medium was replaced every 2 days.

Light microscopy. All cultured cells were examined daily for growth under a phase contrast microscope (Olympus Corporation, Tokyo, Japan).

Monitoring of secretion function. Each culture of cells was maintained in an incubator at 37˚C with 5% humidified CO_2. Supernatant was collected when cells reached 80-90% confluence (within 2 days), and was conserved at -70˚C following centrifugation at 1,000 x g for 5 min at room temperature. The samples were thawed at 4˚C for 24 h, then tested with the rat BDNF, GDNF, NGF and CNTF ELISA kits at room temperature. The values were expressed in pg/ml. At least four samples were analyzed for each culture.

Data analysis. Data were expressed as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 5.1 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was performed for statistical evaluation, followed by the Newman-Keuls multiple comparisons test for stepwise multiple comparisons among the culture groups. $P<0.05$ was considered to indicate statistical significance.

Results

Morphology of cultures. There were no obvious differences in morphological features among the primary cultured, first passage, second passage and cryopreserved-thawed cells under phase contrast microscopy. Choroid plexus epithelial cells grew in fusiform or exhibited polygon shape prior to fusion (Fig. 1).

Change of neurotrophic factors. First passage and cryopreserved-thawed cells secreted less BDNF and CNTF compared with primary cultured cells and increased levels of these two factors compared with second passage cells, and increased levels of GDNF and NGF compared with primary cultured and second passage cells (Fig. 2), with the differences deemed to be significant ($P<0.05$). These results suggested that first passage culture could decrease secretion of the neurotrophic factors BDNF and CNTF but increase that of NGF and GDNF, while second passage culture diminished secretion of all four neurotrophic factors. Cryopreserved-thawed cells

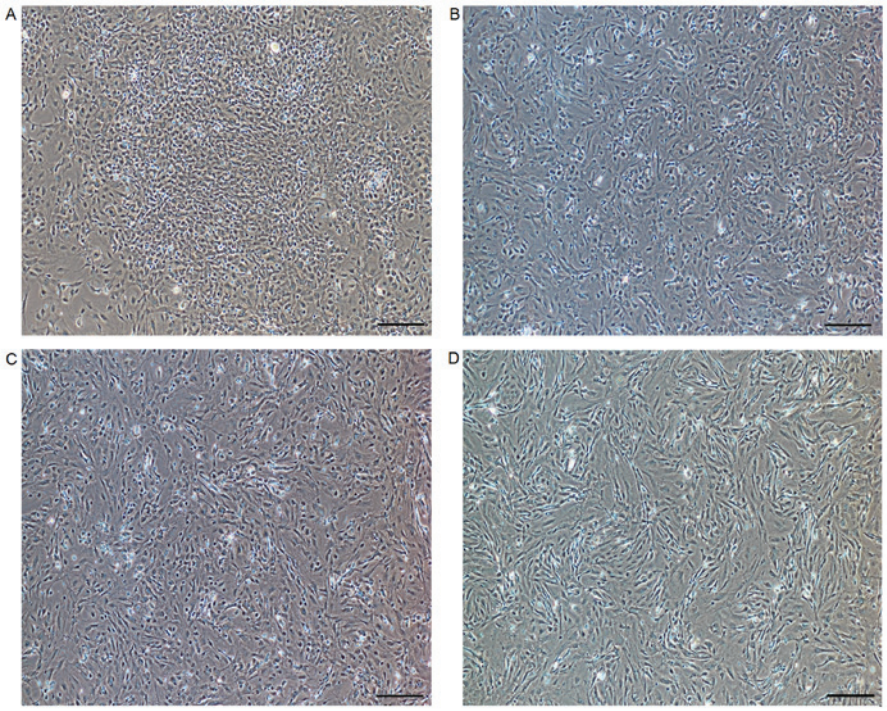

Figure 1. Choroid plexus epithelial cells grew in fusiform or with polygon morphology prior to fusion when observed by phase contrast microscopy (magnification, x100; scale bar, 200 μm). Representative images are shown of (A) primary culture, (B) first passage culture, (C) cryopreserved-thawed cells and (D) second passage culture.

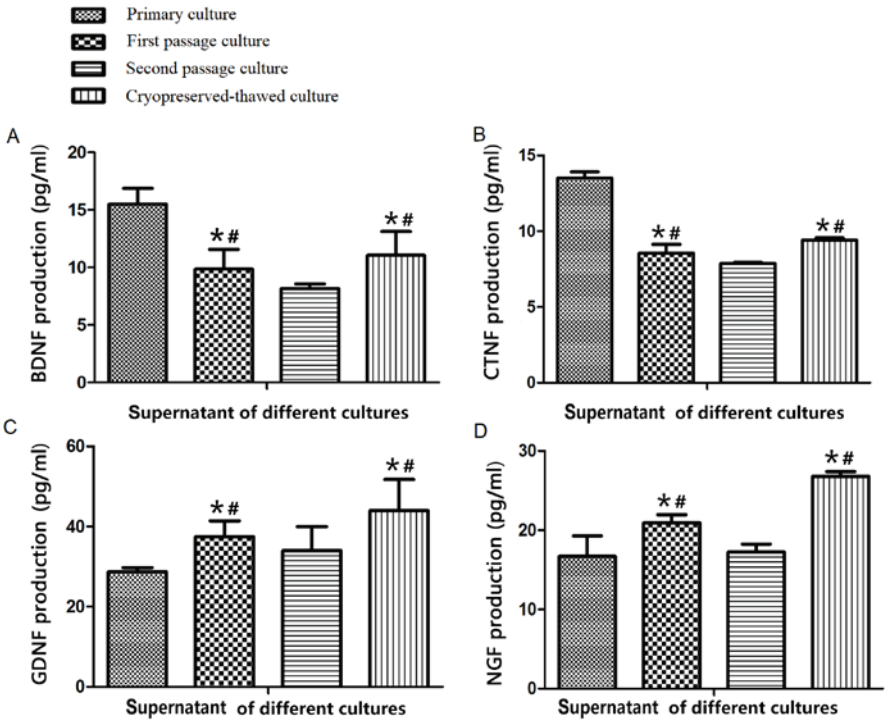

Figure 2. Release of BDNF, CNTF, GDNF and NGF into the cell supernatant of different cultures. Graphical representation of (A) BDNF, (B) CNTF, (C) GDNF and (D) NGF secretion into the culture supernatant is shown. *P<0.05 vs. primary culture; #P<0.05 vs. second passage culture. BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; NGF, nerve growth factor.

secreted the four factors at similar levels to first passage cells (P>0.05), indicating that cryopreservation did not weaken the function of choroid plexus epithelial cells in secreting neurotrophic factors.

Discussion

The present in vitro study demonstrated, to the best of our knowledge for the first time, the effects of passage culture

and cryopreservation on the function of choroid plexus epithelial cells in secreting the neurotrophic factors BDNF, GDNF, NGF and CNTF. The levels of NGF and GDNF released by first passage and cryopreserved-thawed cells were higher compared with those from primary cultured cells, indicating that the function of the cells in secreting certain neurotrophic factors may be enhanced by first passage culture and cryopreservation. In the present study, supernatant was collected when cultured cells reached 80-90% confluence. At this time point, secretion of neurotrophic factors from choroid plexus epithelial cells was expected to be maximal, as the quantity of cells was sufficient and there was no contact inhibition, and the data should therefore reflect the total secretion function.

Primary cultures have been used in transplantation studies for spinal cord injury therapy (8,13). It has been revealed that neurotrophins are key to the therapeutic effects (14). BDNF, GDNF and NGF are important for the survival, maintenance and regeneration of specific neuronal populations in the adult brain and spinal cord (15,16). CNTF, which is widely distributed in the neuronal system, has nutritional function (17,18). Transplantation of primary cultures of choroid plexus epithelial cells has been examined in rats with spinal cord injury, as a therapy to accelerate locomotor improvement and tissue repair including axonal extension in spinal cord lesions (8,19). Studies suggest that the therapeutic effect may be linked with the cultured cells' function of secreting neurotrophic factors including BDNF, GDNF, NGF and CNTF (4,20). However, cells are difficult to obtain in primary culture and the cell source for transplantation is thus limited. If passage cultured and cryopreserved cells can be demonstrated to secrete neurotrophic factors in high quantities, they maybe used for transplantation and the shortage of choroid plexus epithelial cells can be overcome.

The cells in the current study were passage cultured up to second passage and neurotrophic factors were quantified for only three generations. Therefore, the data do not offer a trend to elucidate the effect of continuous passage culture. In future study, the passage cultured cells and cryopreserved choroids plexus epithelial cells should be applied in spinal cord injury models, to determine the efficacy of both cell transplantation and purified neurotrophic factors.

In conclusion, first passage cultured and cryopreserved-thawed choroid plexus epithelial cells may secrete high levels of neurotrophic factors, among which the levels of NGF and GDNF may be higher than those secreted by primary cultured cells. Second passage cells secrete comparatively less neurotrophic factors. Based on the present results, first passage and cryopreserved-thawed cells may have therapeutic potency in the treatment of neural degenerative diseases and could be adopted in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FZ, XYD, FW, XHL and YHL performed the experiments and analyzed the data. FZ wrote the original manuscript. SLH designed the research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols for animal experiments were approved by the Xi'an Jiaotong University Animal Experimentation Committee (Xi'an, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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