International Journal of Pediatric Otorhinolaryngology 73 (2009) 969–973

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: www.elsevier.com/locate/ijporl

Effect of cryopreservation on proliferative features of neural progenitor cells derived from olfactory bulb of embryonic rat

Tao Xue [a,1], Li Wei [b,1], Ran-hong Zheng [c,1], Li Qiao [a], Jian-hua Qiu [a,*], Fu-quan Chen [a], Lian-jun Lu [a], Ding-jun Zha [a], Yu Han [a], Jun Chen [a]

[a] Department of Otolaryngology, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China
[b] Department of Obstetrics and Gynecology, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China
[c] Shaanxi Provincial Health Department, Xi'an 710032, China

ARTICLE INFO

Article history:
Received 6 January 2009
Received in revised form 18 March 2009
Accepted 20 March 2009
Available online 23 April 2009

Keywords:
Neural precursor cell
Cryopreservation
Olfactory bulb
Embryonic

ABSTRACT

Objective: Stem cell research offers unique opportunities for developing new medical therapies for devastating diseases and a new way to explore fundamental questions of biology. The use of olfactory bulb neural progenitor cells for transplantation requires efficient recovery methods and cryopreservation procedures. The purpose of this study was to determine cryopreservation techniques for neural progenitor cells derived from olfactory bulb (OB NPCs) of embryonic rat.

Methods: Initially, we compared the survival rates of cryopreserved OB NPCs using three cryoprotectants: dimethyl sulfoxide (DMSO), ethylene glycol (EG) and glycerol with or without 10% FBS. Cells were held at liquid nitrogen (−186 °C) for 7 days ("short-term storage") or 6 months ("long-term storage"). We assessed OB NPCs recovery efficiency after freezing and thawing by viability testing, colony-forming ability and immunocytochemistry under different conditions.

Results: The survival rate of cryopreserved–thawed OB NPCs was estimated by counting colony numbers under a stereomicroscope. No significant difference in survival rate was observed between cryoprotectants.

Conclusions: These observations indicate that cryopreservation of OB NPCs is possible for up to 6 months in optimal conditions without losing proliferation activity.

1. Introduction

The replacement of lost or damaged neurons by neural progenitor cells (NPCs) or neural stem cells (NSCs) is a distant hope for the treatment of many neurological disorders. Multipotent NSCs/NPCs can be found in the embryonic, neonatal, and adult mammalian central nervous system (CNS) [1]. NSCs are multipotent and can differentiate into different mature cell types. In contrast, NPCs have a more restricted lineage commitment and lower self-renewal capacity than NSCs. Both NSCs and NPCs are believed to play an important role in the maintenance of adult tissues by generating new cells that replace those lost as a result of injury or normal wear and tear [2,3]. Multipotential progenitor with stem cell features can be isolated from the SVZ but also from the entire rostral extension, including the distal portion within the olfactory bulb (OB) [4,5].

Recently, the regenerative capacity of the olfactory system has attracted attention. Olfactory ensheathing cells (OECs) from olfactory bulbs have been used to provide limited axonal regeneration [6–8] and repair demyelinated regions of the CNS [9–11]. The clinical implications of OB NPCs are potentially profound. In practice, long-term expansion and reliable differentiation of neural progenitor cells have proven somewhat difficult. Cryopreservation may be a prerequisite for quality assurance, storage, and distribution required for tissue that shall be used clinically. Therefore, development of appropriate cryopreservation techniques is required. Cryopreservation of NSCs has been done using dimethyl sulfoxide (DMSO). However, DMSO is known to be toxic, with side-effects after prolonged exposure [12,13]. Our recent results [14] imply that neural progenitor cells derived from the olfactory neuroepithelium in adult rats might be cryopreserved for periods of >3 months without losing their proliferative and multipotency activities. Now, in an attempt to find a less-toxic compound able to replace DMSO and exerting less toxicity, other protective agents were

* Corresponding author. Tel.: +86 29 84775381; fax: +86 29 83224744.
E-mail addresses: qiujh@fmmu.edu.cn, xuetao@fmmu.edu.cn, xuetao75123@gmail.com (J.-h. Qiu).
[1] These authors contributed equally to this study.

0165-5876/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ijporl.2009.03.017

investigated. In this paper we show that OB NPC cultures derived from embryonic rat can be cryopreserved. The ability to cryopreserve OB NPC greatly simplifies the use of this cell culture system.

2. Materials and methods

2.1. Animals and reagents

OB NPCs were obtained from the olfactory bulbs at Sprague–Dawley rat embryonic days 14–16. Animals were housed in a temperature and humidity controlled room that was maintained on a 12-h light/dark cycle and had free access to food and water. All protocols of experiments followed were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University, Xi'an, People's Republic of China.

Fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) were purchased from PeproTech (USA) and B27 purchased from Invitrogen (CA). The CCK-8 kit was purchased from Dojindo Laboratories (JPN). Others reagents for tissue culture were purchased from HyClone (USA) and Sigma (USA).

2.2. OB NPCs isolation and culture

Upon the removal of brain, the olfactory bulbs were dissected at the level marked in Fig. 1A of E15 rat brain. Cells were obtained by mechanical dissociation of dissected and pooled olfactory bulbs, followed by mild trypsinization. OB NPCs were seeded in Dulbecco's modified eagle's medium/nutrient mixture F12 (DMEM/F12, Invitrogen Corp., CA, USA), containing penicillin (50 U/ml, Sigma, USA) and streptomycin (50 μg/ml, Sigma) at 37 °C in 5% CO_2 and 95% O_2. B27 (GIBCO, USA), basic fibroblast growth factor (bFGF, Cytolab, Israel) and epidermal growth factor (EGF, Cytolab) (20 ng/ml each) were added every other day. Cells growing as floating aggregates or "spheres" were passaged every 4–7 days before the center became necrotic cells were grown in uncoated plastic flasks during the primary culture as free-floating clusters (neurospheres). Neural stem/progenitor cell populations were obtained from neurospheres after at least 10 passages at 37 °C, in a humidified 5% CO_2 atmosphere. All procedures were prepared as described previously [15,16].

To initiate differentiation, after FGF-2 and EGF were removed, colonies of OB NPCs were plated for 10 days on glass coverslips precoated with 0.1 mg/ml poly-L-lysine in DMEM/F12/B27 containing 10% FBS at a density of 100,000 cells cm^{-2}. The cells were then fixed with 4% paraformaldehyde to assess whether these dividing cells could differentiate to neurons, astrocytes, and oligodendrocytes by immunocytochemistry.

2.3. Cryopreservation techniques and post-thawed

For the cryopreservation of OB NPCs populations, cells were prepared as described above. After at least 10 passages of neurospheres, the samples were resuspended in special cryoprotectants, then stored in a freezer at −70 °C, 24 h later the parallel samples were transferred to liquid nitrogen (−196 °C). Here, six different cryoprotectants were applied, which were mainly made up with serum-free expansion medium. Moreover, each was supplemented with different cryoprotective additives (DMSO, glycerol, and ethylene glycol), as well as with or without 10% FBS. After the 3 days or 3 months of freezing period, the cells were taken from liquid nitrogen, thawed rapidly in a 37 °C water bath with continuous agitation, then diluted with the culture medium to 10 times of volumes. Four hours later, freezing medium was replaced with the fresh serum-free expansion medium, and neurospheres were maintained as described above.

2.4. Measurement of cell viability and cell proliferation assay

To measure trypan blue exclusion as described [14], the viable cell ratios were calculated according to the following formula: viable cell ratio (%) = (nonstained cells number/total cells number) × 100%. Necrotic cells (percent percent) referred to percentage of trypan blue-positive cells.

To measure CCK-8, fresh or frozen–thawed OB NPCs in the above different cryoprotective additives were monitored via inverted microscope, and their viabilities were detected with a CCK-8 kit to calculate cell survival according to the protocols of CCK-8.

2.5. Clonogenic survival assay

Clonogenicity with different cryoprotectants was determined by measuring colony formation as described [17]. In brief, after the cells were thawed and washed, OB NPCs were adjusted to a final concentration of 5×10^4 ml^{-1} in 9 ml culture medium. The cells were seeded in triplicates in six-well plates. Relative survival value was calculated by assessing the ratio of colony-forming units (CFUs) of secondary neurospheres, which were treated with different cryopreservation techniques and thawed as mentioned before, then exposed to the fresh culture samples for 14 days at 37 °C, in a humidified 5% CO_2 atmosphere.

2.6. Statistics

Results are expressed as mean ± standard deviation. In case of statistically significant differences, Tukey's test was used to determine which groups statistically differed from each other. One-way analysis-of-variance (ANOVA) procedures were employed

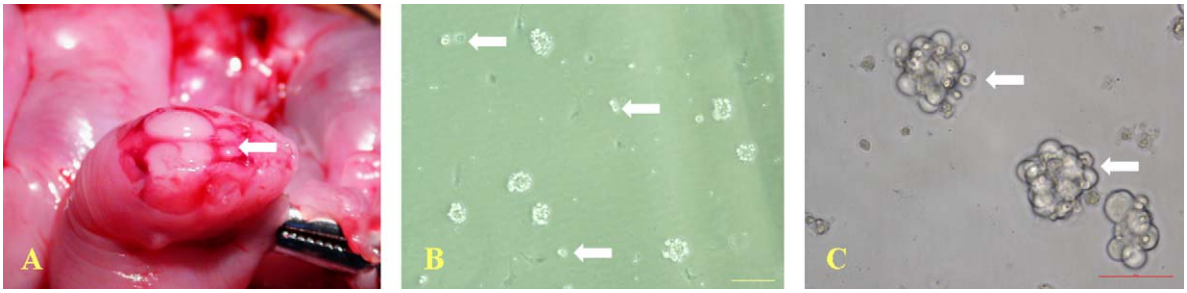

Fig. 1. Cells that respond to FGF-2 plus EGF by proliferating and forming spheres can be isolated from the embryonic olfactory bulb. Cells prepared from the OB of E14.5, E15.5, and E16.5 rat were plated in the presence of 20 ng/ml FGF-2 and 20 ng/ml EGF. (A) View of an E14.5 rat brain. The white arrowheads mark the plane of the OB dissection; (B) 3 days after plating, some rounded cells started to divide. Scale bar corresponds to 100 μm; (C) daily inspection of the cultures showed that the rounded cells divided, forming cell aggregates or spheres, which reach confluence in the presence of the mitogens by the 7th day. Scale bar corresponds to 50 μm.