Research Paper: Isolation and Differentiation of Neural Stem/Progenitor Cells From Subventricular Zone of One Adult Rat

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ABSTRACT

Introduction: In adult mammalian brain, neural stem cells are isolated from both the dentate gyrus and subventricular zone. This study aimed to isolate neural stem cells from adult rat subventricular zone and differentiate them into neurons and astrocytes.

Methods: In this study, the whole brain was removed after full anesthesia and creating cervical dislocation. Under a microscope, subventricular zone was dissected by a coronal incision in optic chiasm zone. Enzymatic digestion was performed using trypsin-EDTA. The isolated cells were cultured in serum free DMEM/F12 medium, containing bFGF (basic Fibroblast Growth Factor) and EGF (Epidermal Growth Factor) growth factors.

Results: Neurospheres were observed five days after culturing. Immunocytochemistry was used to investigate nestin gene expression and identify neural stem cells. Neural stem cells were differentiated in poly-L-lysine coated plates in the absence of growth factors. The expression of GFAP, β tubulin III, and nestin genes were analyzed by RT-PCR. The results of immunocytochemistry confirmed nestin gene expression in the neural stem cells. Phenotype of neurons and astrocytes were observed 5 days after cell culture in differentiation medium. RT-PCR analysis revealed the expression of GFAP and β tubulin III genes.

Conclusion: The results of this research show that only one rat brain is needed for neural stem cells isolation and differentiation to neurons and astrocytes.

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1. Introduction

Previously, it was believed that stem cells could only be found in the tissues more susceptible to damage and cell death, and since central nervous system has shown no significant neuronal death and ability to regenerate, so the presence of stem cells in this system seemed to be improbable and unnecessary. However in 1992, the presence of stem cells was demonstrated in the central nervous system of adult mammals with the ability to make new neurons [1]. Recent research suggests that steady nerve regeneration occurs in some restricted areas of the central nervous system. Two well-known areas in the brain of adult mammals include brain subventricular zone and hippocampal dentate gyrus [2, 3].

Neural Stem Cells (NSCs) are pluripotent progenitor cells with self-renewal activity that generate astrocytes and oligodendrocytes in vitro, in addition to neurons, as well [4, 5]. Due to lack of specific surface markers for the NSCs, it is possible to isolate these cells in vitro using physiological characteristics. In 1992, Reynolds and Weiss developed a method called neurosphere assay to isolate these cells [1].

Neural stem cell transplantation in the last two decades has been proposed as a selective therapeutic approach to brain damage, neurodegenerative disorders, and certain neurological disorders. Successful treatment in some patients with Parkinson disease using stem cells has held promises for researchers to use NSC to treat or regenerate the brain damages [6, 7].

Recent studies have shown that transplantation of neural progenitor cells derived from the adult brain to animal model with spinal cord injury has led to the renewal of myelination in the cell, which is similar to the myelin formation process in Schwann cells [8]. All of these invaluable features of NSCs make them excellent candidates for use in the treatment of central nervous system associated diseases.

2. Materials and Methods

Culture medium preparation

DMEM/F12 containing GlutaMAX was dissolved in distilled water. Then sodium bicarbonate and HEPES were added and thoroughly mixed. A mixture of 100 units per mL penicillin (to prevent the growth of Gram-positive bacteria) and 100 mg/ml streptomycin (to prevent the growth of Gram-negative bacteria) were added to the culture medium. The resulting solution was transferred under the hood and was sterilized using a syringe filter.

Rat brain subventricular zone dissection

In this study, 6- to 8-week-male Wistar rats were anesthetized by intraperitoneal injection of ketamine and xylazine mixture with a ratio of 8 to 1. After cervical dislocation, the head was cut off from the upper cervical spine. Then, a sagittal incision was made in the skin on the head and the skull bones were visible by pushing the skin on either side of the incision. The head was fixed by taking edges of the incised skin between two fingers and a coronal incision was made in the orbital bones between the eye sockets. A sagittal incision was made along the sagittal suture from the foramen magnum to the previous coronal incision between the two eyeballs using fine sharp scissors. The incised bone edges on each side were pulled up and out using a curved-tip forceps to expose the brain. Then the dissected brain was moved into the falcon tube, containing PBS using a curved-tip spatula.

To dissect and separate the subventricular zone, the brain was placed on the dorsal surface after removal of the olfactory bulb. The brain was fixed in cerebellum part using a forceps and a coronal incision was made in the optic chiasm. The caudal part of the brain was removed and the rostral part was used for isolating subventricular zone. The forebrain was placed in a standing position and on the frontal pole of the brain at the incision site of olfactory bulb, so that the lateral ventricles sections were the upward. First, the septum between two ventricles of the brain was removed and discarded. Then a thin layer of the outer wall of the lateral ventricles was taken and placed in sterile Petri dishes. Isolated tissue of subventricular zone was moved into laminar hood.

Isolation and culture of NSC from the subventricular zone

Under the laminar hood, the subventricular zone was split into small pieces with a scalpel over a period of two minutes. Then, the tissue was mixed by one mL of trypsin-EDTA, after pippetage transferred into the 15-ml falcon tube, and incubated for 7 minutes at 37°C. The effect of trypsin was neutralized by adding medium on trypsin and doing pippetage. The falcon tube was centrifuged at 700 rpm for 5 minutes. The supernatant was discarded and about 200 µL of DMEM/F12 medium was added to the cells and pippetage was performed gently up to 7 times. The medium was then added into falcon tube to reach the final volume of 10 mL. The mixture containing brain tissue cells and remnants were passed through the 40-µm cell strainer.

The cells passed through the filter and undigested tissue remnants were filtered. The medium containing filtered

cells were recentrifuged with the above conditions. The supernatant was discarded and pippetage was performed for cells in DMEM/F12 medium containing GlutaMAX and HEPES buffer. The above medium was added 1 µL/ml of 0.2% heparin, 10 ng/ml of bFGF (basic Fibroblast Growth Factor), 20 ng/ml of EGF (Epidermal Growth Factor) and 2% B27. The isolated cells of the brain were transferred into a T25 cell culture flask. The flask was incubated at 37°C and 5% CO_2. Part of the medium was removed after three days and fresh medium was added.

Identification of neural stem cells using immunocytochemistry

The cells were transferred on poly-L-lysine coated cover slips and culture medium was added. After 24 hours, neurospheres were attached to the bottom of cover slips, and got ready for immunocytochemistry test. To carry out immunocytochemistry, the cells were fixed firstly using 4% paraformaldehyde for 30 minutes, and then washed with PBS three times and each time for 5 minutes. Blocking solution (a mixture of 10% normal serum and 0.3% Triton X-100 in PBS) was added and incubated for one hour. The cells were incubated overnight in a mouse anti-nestin antibody at a concentration of 0.01 at 4°C. The cells were then washed with PBS three times and each time for 5 minutes, and finally incubated with FITC-conjugated secondary antibody at a concentration of 1/50 for one hour, and after washing with PBS, they were examined under the fluorescent microscope.

Differentiation of neural stem cells

NSCs with a density of 1×10^4 cells/cm^2 were cultured on the poly-L-lysine coated plate in the medium, containing 20 ng of EGF and 10 ng of bFGF. After reaching the cell density by over 70%, the cell culture medium, containing 5 ng of bFGF and without the addition of EGF was replaced. After 48 hours, this medium was replaced with medium without the growth factors.

RNA isolation and polymerase chain reaction

Total RNA was isolated using RNA isolation kit (Roche). DNase I enzyme was recruited to enhance the purity and quality of RNA. The cDNA related to the differentiated cells mRNA was synthesized using reverse transcriptase (RT) enzyme. Two µL of RT product with 25 µL of Master Mix 2X (Fermentas) were poured in the microtube. Then 50 pmol of each primers (Table 1) was poured into each microtube, and the final volume was brought to 50 µL using nuclease free water. The microtube content was well mixed and then placed inside the thermocycler device. The polymerase chain reaction was performed according to program shown in Table 2. GAPDH that is a housekeeping gene was used as internal controls in this test.

3. Results

The results of this study showed that the NSCs from adult rat subventricular zone cultured in serum-free medium, containing bFGF and EGF growth factors would lead to the formation of neurospheres. Neurosphere assay is the proper method for isolating these cells from other cells. Isolation of subventricular zone under the loop microscope with appropriate magnification caused an increase in the efficiency of creating pure NSCs so that the primary colonies of neurosphere were seen gradually after 5 days. Colony size was enlarged rapidly by replacing part of the medium at this stage and reached to 150-200 µm within 7 to 9 days and became ready

Table 1. Sequence of primers used in PCR.

Gene	Primer Sequence	Fragment Size (bp)
GFAP	F: 5′-CAGGAAGAGTAAGCAACGGC-3′	205
	R: 5′-GTGGGTATTCACGTGTCCCT-3	
β-tubulin III	F: 5′-TTCATGGTGGAGATGGCCTT-3′	222
	R: 5′-CTAGTCTCCTGCTGCTACCC-3′	
Nestin	F: 5′-ATGAGGGGCAAATCTGGGAA-3′	243
	R: 5′-CCAGGTGGCCTTCTGTAGAA-3′	
GAPDH	F: 5′-TAGGGCTGGAAAATCACTGG-3′	211
	R: 5′-GTATTCATCACCCCCACCAC-3	

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Table 2. Polymerase chain reaction process in thermocycler device.

Step	Temperature	Duration	No of Cycles
Initial denaturation	94°C	5 min	1
Denaturation	72°C	30 s	35
Annealing	59°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	1

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for passage. After the first passage, purity rate of neurospheres increased and the number of colonies formed in the flask was far more (Figure 1).

Using immunocytochemistry assay, anti-nestin antibody was used to investigate and approve the nature of neural stem cells. The expression of anti-nestin antibody showed that the studied cells are NSCs (Figure 2). Cell phenotype was changed 5 days after culturing the NSC in poly-L-lysine-coated plates, and differentiated cells were observed (Figure 3). The results of RT-PCR showed that the GFAP, β-tubulin III, nestin, and GAPDH genes were expressed in differentiated cells (Figure 4). GFAP and β-tubulin III genes expression confirmed the differentiation of NSC into astrocytes and neuronal cells.

4. Discussion

In 1992, the presence of stem cells was demonstrated in the central nervous system of adult mammals with the ability to make new neurons [1]. These cells can induce neurogenesis in adult mammalian brain in pathological conditions. Hence, neural stem cells are of great importance as a therapeutic approach to repair some of central nervous system disorders [9-11]. Important point in the

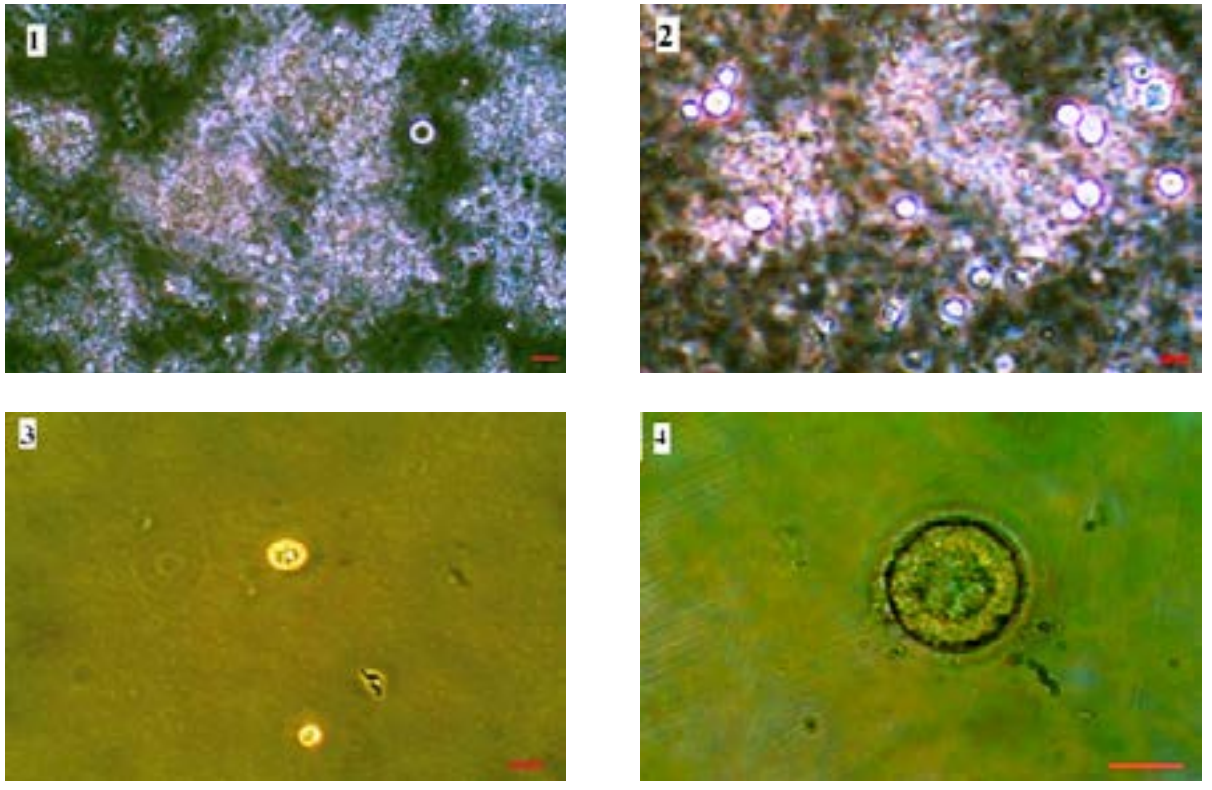

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Figure 1. A phase-contrast image of forming neurospheres within 5 days after culturing (1), on the seventh day (2) and after first passage (3) with a magnification of 200×. A mature neurosphere seven days after the first passage of cells is displayed with a magnification of 400× (4). Scale bar=100 µm.

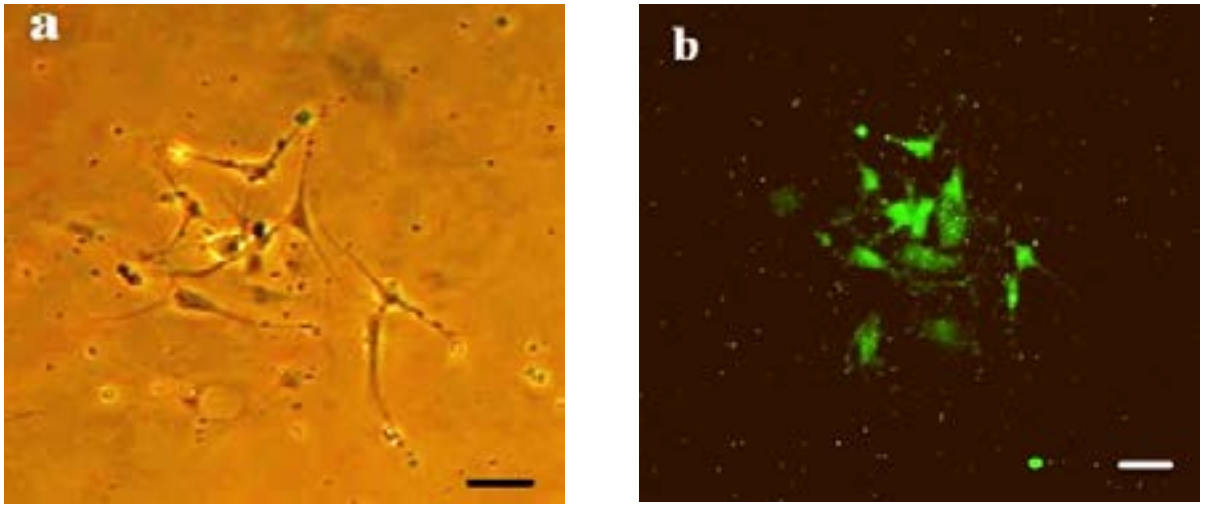

Figure 2. A phase-contrast image of the NSC cultured on poly-L-lysine coated cover slip (a); A fluorescent image of nestin-positive cells (b).

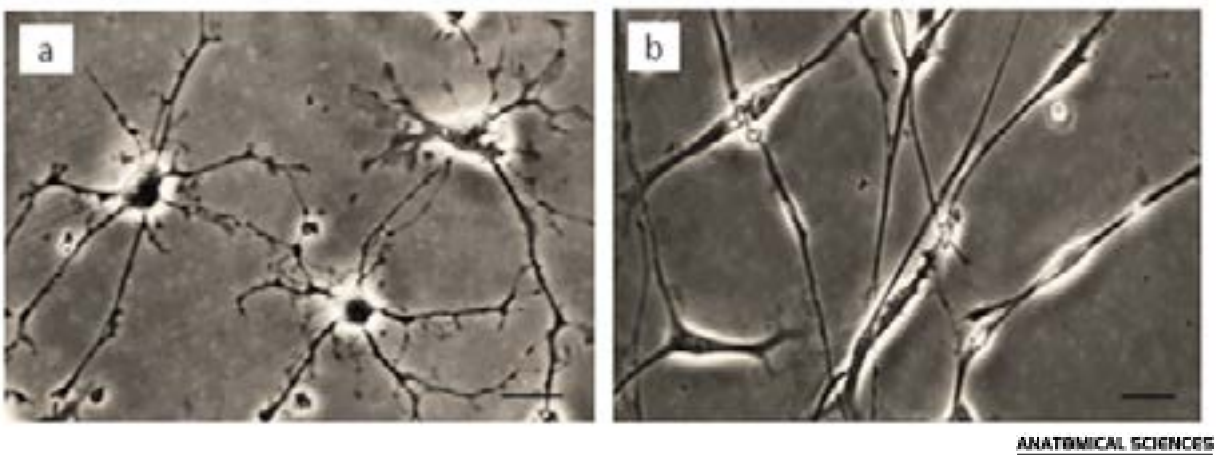

Figure 3. Phase-contrast microscopic images of differentiated cells; Phenotypes of astrocytes(a); Bipolar and multipolar neurons and intercellular synapses (b).

use of these cells is how to extract and isolate these cells from other brain cells. Accurate isolation of subventricular zone from other areas of the brain increases efficiency of neurosphere production. Selection of tissue dissection method is one of the factors, which increases the efficiency of stem cells. The use of 0.05% trypsin-EDTA for enzymatic digestions and extraction of NSC from the SVZ is more suitable than other methods.

Neural stem cells after isolation at serum-free medium in the presence of bFGF and EGF appear as floating spheres, called neurosphere. Other cells will die within few days in serum free medium [12]. Excessive increase in the size of the neurosphere would result in their adhesion to the culture plate, leading to gradual differentiation of the cells. The neurosphere growth is always accompanied by the presence of a small number of differentiated cells. Therefore, the use of methods such as immunocytochemistry seems necessary to confirm the nature of stem cells [13, 14].

In this study, after confirming the nature of neural stem cells, these cells were differentiated into astrocytes and neuronal cells in vitro. Differentiated cells showed well the astrocytes and neuronal cells phenotype. The expression of GFAP (astrocytes-specific gene) and β-tubulin III (neuron-specific gene) genes proves NSC differentiation into astrocytes and neuronal cells. High expression of GFAP gene indicates that the majority of NSC in differentiation medium differentiates into the astrocytes. Low expression of nestin gene that reflects the nature of the NSC is due to the presence of NSCs in the media that are still undifferentiated. Low expression of this gene suggests that most cells have differentiated into other cells.

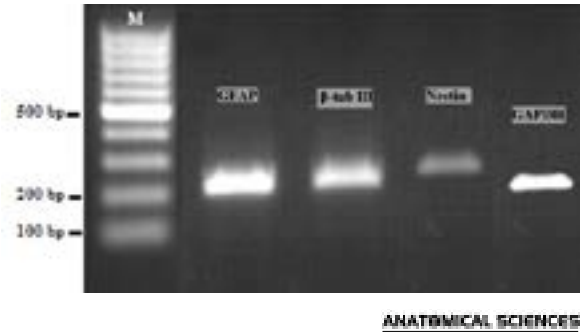

Figure 4. Electrophoresis of RT-PCR product. 100 bp DNA ladder marker has been used (M).

Azari and colleagues have conducted a similar study. In their study, the brains of several mice were simultaneously used to extract NSCs and exam their differentiation. In the study, NSCs were isolated using neurosphere assay and formed neurospheres [15]. In our study, the cells were isolated from only one rat brain. Although isolating these cells is much more difficult using one brain and requires microdissection and accurate enzymatic digestion, this protocol on the brain provides the possibility of applying in limited cases such as transgenic mice or genetic studies [16]. In vitro extraction of NSCs and their differentiation into neurons and glial cells and autologous transplantation of the differentiated cells could be a big step forward in the treatment of the CNS diseases.

The results of this research suggest the possibility of isolation and culture of the rat neural stem cell by applying precise microdissection and proper dissection for subventricular zone tissue. These cells differentiate into astrocytes and neuronal cells in differentiation medium. The expression of GFAP and β-tubulin III genes proves NSC differentiation.

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Conflict of Interest

All authors certify that this manuscript has neither been published in whole nor in part nor being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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