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The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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ORIGINAL ARTICLE

Basic Study

Culture and identification of neonatal rat brain-derived neural stem cells

Qing-Zhong Zhou, Xiao-Lan Feng, Xu-Feng Jia, Nurul Huda Binti Mohd Nor, Mohd Hezery Bin Harun, Da-Xiong Feng, Wan Aliaa Wan Sulaiman

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Abstract

BACKGROUND

Timing of passaging, passage number, passaging approaches and methods for cell identification are critical factors influencing the quality of neural stem cells (NSCs) culture. How to effectively culture and identify NSCs is a continuous interest in NSCs study while these factors are comprehensively considered.

AIM

To establish a simplified and efficient method for culture and identification of neonatal rat brain-derived NSCs.

METHODS

First, curved tip operating scissors were used to dissect brain tissues from new born rats (2 to 3 d) and the brain tissues were cut into approximately 1 mm^3

sections. Filter the single cell suspension through a nylon mesh (200-mesh) and culture the sections in suspensions. Passaging was conducted with TrypL™ Express combined with mechanical tapping and pipetting techniques. Second, identify the 5th generation of passaged NSCs as well as the revived NSCs from cryopreservation. BrdU incorporation method was used to detect self-renew and proliferation capabilities of cells. Different NSCs specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies) were used to identify NSCs specific surface markers and muti-differentiation capabilities by immunofluorescence staining.

RESULTS

Brain derived cells from newborn rats (2 to 3 d) proliferate and aggregate into spherical-shaped clusters with sustained continuous and stable passaging. When BrdU was incorporated into the 5th generation of passaged cells, positive BrdU cells and nestin cells were observed by immunofluorescence staining. After induction of dissociation using 5% fetal bovine serum, positive NF200, NSE and GFAP cells were observed by immunofluorescence staining.

CONCLUSION

This is a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification.

Key Words: Neonatal rats; Brain-derived neural stem cells; Culture; Identification

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Core Tip: How to harvest sufficient neural stem cells (NSCs) is a basic requirement for the study and clinical application of NSCs. This study describes a simplified and efficient method for neonatal rat brain-derived NSC culture and identification comprehensively considering the influencing factors including timing of passaging, passage number, passaging approaches and methods for cell identification. It demonstrates that combination of TrypL™ Express and mechanical tapping and pipetting techniques makes a more efficient way of passaging. The optimal timing for NSC passage is on the fourth to fifth day of primary or passage NSC culture.

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INTRODUCTION

Spinal cord injuries (SCIs) are associated with high morbidity, disability and medical costs around the world[1-3]. Prevention, treatment and rehabilitation of SCIs remains one of the global health issues. Pathophysiological basis for sensory, motor and sphincter dysfunction below the neurological level of injury is the substantial variations, necrosis and loss of neurons along with ruptured tracts of spinal cord as well as demyelination induced by primary and/or secondary SCIs[4,5]. Therefore, the research fucus on the key to promoting recovery of function following SCIs falls on how to replenish the lost neurons and boost the regeneration of axis cylinder and myelin sheath. It should be noted that neural stem cells (NSCs) have the capacity to self-renew and proliferate, and differentiate into nerve cells of central nervous system including neuron, astrocyte and oligodendrocyte[6]. Hence, by replenishing neurons, forming axis cylinder, increasing oligodendrocyte and reconstructing medullary sheath and then remolding the injured structure and function of spinal cord, NSCs replacement as a possible therapeutic approach could bring hope to SCI patients. In view that rapid and simplified accesses to a large number of pure NSCs for research are needed, it is necessary to develop a method for NSC amplification, culture, purification and identification *in vitro* to provide sufficient-necessary condition for further investigation of NSCs.

The present study cultured, passaged and harvested large amounts of high pure NSCs with growth factor containing serum-free medium using TrypLE™ Express digestion method combined with mechanical disassociation method[4,7,8], which provide the platform for the further study of NSCs.

MATERIALS AND METHODS

Rats

In this study, Brain-derived NSCs were abstracted from several two-to-three-day old newborn SD rats provided by Southwest Medical University Animal Resource Center.

Reagents

Hyclone DEME/F12 (1:1); Hyclone standard fetal bovine serum; Peprotech EGF (20 μg); Peprotech bFGF (10 μg); Gibco B27 Plus Supplement (50×); Gibco TrypL™ Express (1×); rabbit anti-nestin polyclonal antibody (Wuhan Boster Biological Technology, Ltd.); mouse anti-NSE monoclonal antibody and mouse anti-GFAP monoclonal antibody (Wuhan Boster Biological Technology, Ltd.); RBITC-labeled goat anti-mouse IgG (Beijing Biosynthesis Biotechnology Co., Ltd.); RBITC-labeled goat anti-rabbit IgG (Beijing Biosynthesis Biotechnology Co., Ltd.).

Preparation of serum free medium for NSC culture

Take 100 mL of DMEM/F12 (1:1) and add EGF (20 ng/mL), bFGF (20 ng/mL), B27 Plus Supplement (2%), glutamine (2 mmol/L) and 1 mL of penicillin-streptomycin solution (100 U/mL). NaOH filtered with 0.22 μm microporous membrane filter were used to adjust PH of the solution between 7.2 and 7.4. After preparation, the medium was stored at 4°C. Serum free medium (SFM) should be kept fresh. A weekly dosage of 100 mL for on preparation is generally recommended.

Primary culture, passage, cryopreservation, resuscitation and identification of NCSs

Preparation of extracts and primary NSC culture: Newborn SD rats (2–3 d old) were selected and immersed in 75% alcohol for 10 min for disinfection. In supine position, the rats were fixed to foam board and placed on the clean bench with cranial cavity exposed. Brain tissues were isolated and placed in sterilization incubators containing phosphate-buffered saline (PBS) (0.01 M, PH 7.2, pre-warmed to 37°C, 10 min, the same below) for washing to remove blood stains on the surface. Then pia mater as well as choroid plexus tissues were removed under a dissecting microscope. The dissected brain tissues were washed and transferred to a new sterilization incubator. Using curved tip operating scissors, dissected brain tissues were cut into approximately 1 mm^3 sections and were transferred to centrifuge tubes (10 mL, the same below) using sterile pipettes. Centrifuge the sections at 1000 rpm for 1 min and remove the supernatant. Save the cerebral cortex in three separate tubes, each containing 1 mL of mixed enzyme, shake them gently for 5 min in a 37°C water-bath and centrifuge them at 1000 rpm for 5 min, and then remove the supernatant. Add 5% fetal bovine serum, pipette and tap gently 10 times using pipettes, shake gently in a 37°C water-bath, centrifuge for 5 min at 1800 rpm, and remove the supernatant. Then add DMEM/F12 (1:1), pipette and tap gently 20 times using pipettes, filter using stainless-steel cell strainers (200-mesh), centrifuge for 5 min at 1000 rpm and remove the supernatant. Use 0.5 mL of SFM to resuspend cells. Pipette and tap the cells gently and form single-cell suspensions. Seed the cells at a rate of 1×10^6/mL (cell density determined by trypan blue method excluding dead cells) in 25 mL glass culture flasks and cultured in carbon dioxide constant temperature incubators (stable temperatures of 37°C, saturated humidity, a CO_2 level of 5%). Centrifuge the cells for 5 min at 1000 rpm every other day or every two days depending on how the cells developed and refresh half of the media with fresh growth media. Approximately, four or five days later, initiate passaging. Observe and record cell growth status under inverted phase contrast microscope every day including medium color, impurities in medium, cell refraction, density, size of neurosphere formation and neurosphere refraction.

Passaging of NSCs: After four to five days of primary culture, which is dependent on the growth process of cell and neurospheres, gather all neurospheres and culture solution and place in a 15 mL cone shaped tube, centrifuge for 5 min at 1000 rpm and remove the supernatant. Add 1 mL of TrypLE™, pipette cells gently using pipettes, shake them gently for 5 min in a 37°C water-bath and centrifuge them at 1800 rpm for 5 min, and then remove the supernatant. Add 0.5 mL of SFM to resuspend cells. Using 1000 μL pipettes, tap the cells gently 10 times (for 200 μL pipettes, approximately 20 times). Seed the cells at a rate of 1×10^6/mL (cell density determined by trypan blue method excluding dead cells) in a 25 mL glass culture flask and cultured in the above described constant temperature incubators (stable temperatures of 37°C, saturated humidity, a CO_2 level of 5%).

Cryopreservation and resuscitation of NSCs: Add 1 mL of freshly prepared cell freezing medium (DMEM/F12:fetal bovine serum:DMSO = 7:2:1) to the single-cell suspensions. Adjust the cell density to 5×10^6/mL, and preserve the suspension in a 4°C refrigerator for three hours. Place the cryovials in a small plastic storage box and keep the box immediately in a −80°C gas-phase liquid nitrogen freezer overnight and finally cryopreserve the cryovials in a liquid-phase liquid nitrogen freezer. For cell resuscitation, remove cryovials from the liquid-phase liquid nitrogen freezer and place the cryovials in a 37°C water-bath for rapid melting. Transfer the cells to centrifuge tubes and add 4 mL of basal media. Centrifuge the cells for 5 min at 1000 rpm and remove the supernatant. Add SFM and resuspend cells. Seed the cells at a rate of 1×10^6/mL (cell density determined by trypan blue method excluding dead cells) in 25 mL glass culture flasks and cultured in the above described constant temperature incubators.

Identification of NCSs: Identify the 5th generation of passaged NSCs and the revived NSCs from cryopreservation. Use bromodeoxyuridine (BrdU) incorporation method to detect the self-renew and proliferation abilities of cells. Use diverse neurocyte-specific antibodies such as anti-nestin, anti-NF200, anti-NSE and anti-GFAP antibodies to examine NSC specific surface markers and muti-differentiation capabilities by immunofluorescence staining.

BrdU incorporation method for the detection of the proliferation of NSCs: (1) At 24 h after the culture of 5th generation of passaged NSCs, add BrdU to SFM to make BrdU reach a concentration of 10 μmol/L. Continue to culture the cells for five days and seed the NSCs cultured in suspensions at a rate of 1 × 10^6/mL (500 μL) in a carbon dioxide constant temperature incubator containing 24-well culture plates with polylysine coated coverslips (stable temperatures of 37°C, moderate saturated humidity, a CO_2 level of 5%) for four hours; (2) Draw the solution and gently wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (3) Add 4% paraformaldehyde for a fixation for 15 min at room temperatures; (4) Draw the stationary fluids and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (5) Use 37°C blocking buffer (10% sheep serum and 0.3% Triton) for 1 h closed incubation (150 μL per well, almost spread the coverslips) and then draw the stationary fluids. Add 150 μL of mouse anti-BrdU primary monoclonal antibody (dilute the fluids with PBS to adjust the concentration to 1:200) and cover coverslips. Use PBS (0.01mol/L, PH7.2) as positive control to replace mouse anti-BrdU primary monoclonal antibody. Continue to culture the cells in a 37°C constant temperature incubator for two hours; (6) Blot fluids in the last step and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (7) Add FITC labeled goat anti-mouse IgG second antibody (dilute the fluids with PBS to adjust the concentration to 1:40) (cells should be protected against exposure to light for each of the following steps). Continue to culture the cells in a 37°C constant temperature incubator for one hour; (8) Blot the second antibody and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (9) Add 20 μL of anti-fluorescence quencher over the slide; and (10) Lift the coverslip using a self-made right-angled tip needle and then press it with the side containing the cells down avoiding creating air bubbles. View the cells under a fluorescence microscope, take photos and make records.

Nestin immunofluorescence staining: (1) Take a small amount of 5th generation of cultured neurosphere containing NSCs and seed them in a carbon dioxide constant temperature incubator containing 24-well culture plates with polylysine coated coverslips (stable temperatures of 37°C, moderate saturated humidity, a CO_2 level of 5%) for four hours; (2) Discard the solution and wash gently 3 × 5 min with PBS; (3) Add 4% paraformaldehyde for a stationary phase for 15 min at room temperatures; (4) Discard the stationary liquid and wash gently 3 × 5 min with PBS; (5) Use 37°C blocking buffer (10% sheep serum and 0.3% Triton) for 1 h closed incubation (150 μL per well, almost spread the coverslips) and then blot the stationary fluids. Add rabbit anti-nestin monoclonal primary antibody (dilute the fluids with PBS solution to adjust the concentration to 1:200). The primary antibody was substituted for PBS solution as a negative control. Continue to culture the cells in a 37°C constant temperature incubator for two hours; (6) Discard the liquid used in the last step and gently wash 3 × 5 min with PBS (0.01 M, PH7.2); (7) Add RBITC labeled goat anti-rabbit IgG second antibody (dilute the fluids with PBS solution to adjust the concentration to 1:40) (avoid light exposure for each of the following steps). Then culture the cells in a 37°C constant temperature incubator for one hour; (8) Blot the second antibody. Wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (9) Add DAPI staining solution (150 μL per well, almost spread the coverslips) for 3 min at room temperatures and then blot the DAPI staining solution. Wash 3 × 5 min with PBS (0.01mol/L, PH7.2). Add 20 μL of anti-fluorescence quencher over the slide; and (10) Lift the coverslip and then press it with the side containing the cells down on the slide containing 20 μL of anti-fluorescence quencher (Be careful to avoid creating air bubbles).

Observe the cells under a fluorescence microscope, take photos and make records. Differentiation of NSCs and immunofluorescence staining of GFAP, NF200 and NSE. (1) Using SFM containing 5% fetal bovine serum, seed the 5th generation of NSCs in 24-well culture plates with polylysine coated coverslips and culture the cells for 5 d; Repeat steps (2), (3) and (4) in the section of BrdU incorporation method for the detection of the proliferation of NSCs or in nestin immunofluorescence staining; (5) Use 37°C blocking buffer (0.3% Triton X-100 and 10% sheep serum) for 1 h closed incubation (150 μL per well, almost spread the coverslips, similarly hereinafter) and then discard the stationary fluids. Add mouse anti-GFAP monoclonal antibody, mouse anti-NSE monoclonal antibody and mouse anti- NF200 monoclonal antibody, respectively (all as primary antibody, dilute the fluids with PBS to adjust the concentration to 1:200), and the primary antibody was substituted for prepared PBS solution as a negative control and then cultured in the above described constant temperature incubators for two hours; (6) Discard the liquid in the last step, and wash 3 × 5 min with prepared PBS; (7) Add RBITC-labeled goat anti-mouse IgG secondary anti-body (dilute the fluids with PBS to adjust the concentration to 1:40), and incubate the cell in a above mentioned incubator for 1 h; Repeat steps (8), (9) and (10) in the section of BrdU incorporation method for the detection of the proliferation of NSCs or in nestin immunofluorescence staining.

RESULTS

Development and identification of NSCs

The first-generation cells derived from newborn SD rats appear small, equal sized, glossy spheres. At 24 h after the primary culture, larger amounts of cells aggregate in the middle of potion of the growth medium. These single floating cells can differentiate and renew (Figure 1A). There are also plenty of glossy, spherical-shaped, adherent single cells at the bottom of the culture bottle. Moreover, many structural looseness, highly refractile, spherical-shaped clusters and membraniform flocculation also exist in the culture medium, which are considered as clumps of dead cells and uneliminated tissues such as blood vessels and pia mater, respectively. At 2 to 3 d after the primary culture, several and even dozens of translucent, highly refractile, spherical-shaped or thyrsiform cell clusters are discovered (Figure 1B). What's more, only a small amount dead cell clusters were left in the culture medium. At four to five days after the primary culture (Figure 1C), the cell mass bulks up to a spherical shape when large numbers of cell clusters aggregate and form translucent slippery spheres under an inverted phase-contrast microscope. However, refractivity at the center of the sphere decreases and few aggregated dead cells are observed. Compared with that of neurospheres at five days after the primary culture, the volume of neurospheres at eight days after the primary culture is obviously big and the refractivity is poor and it shows dark brown in most parts of the sphere center (Figure 1D). Passaging conducted at that time only dissociates neurospheres into smaller cell clumps and few single floating cells rather than single-cell suspensions. It is speculated that this is associated with tight cell-cell junction around the neurospheres. Moreover, many small sized adherent cells are observed at two days after the passaging with small quantity of low density single floating cells. Adherent differentiation occurs in plenty of large volume neurospheres during the continuous culture.

Generally, there are two methods of cell dissociation: mechanical and enzymatic. It is discovered that it is hard to control enzyme concentration and time, which easily lead to cell death. Comparatively, TrypLE™ Express digestion method combined with mechanical disassociation method can harvest sufficient normomorph, uniform size single floating cells with glossy cytoplasm and there are relatively few cell debris and impurities in the culture medium. In addition, medium-sized cloned neurospheres in good shape can be observed in the passaged NSC culturation (Figure 1E). At four hours after the induction of dissociation of 5th generation of passaged NSCs using 5% fetal bovine serum, adherent neurospheres and gradual migration of cells away from neurospheres are observed. At approximately five days after the induction of dissociation, spheres become flattened and form various shaped cells surrounding the neurospheres. At approximately five days after the induction of dissociation, the spheres flatten and make up lots of various shapes of cells. These cells connect to each other into a mesh-structure (Figure 1F).

Identification of NCSs

The incorporation of BrdU into the 5th generation of passaged cells showed positive after BrdU immunofluorescence staining (green fluorescence, Figure 2A) and nestin immunofluorescence staining (red fluorescence, Figure 2B). At five days after the induction of dissociation of 5th generation of passaged cells using 5% fetal bovine serum, NF200 (green fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus, Figure 2C), NSE (red fluorescence, blue fluorescent obtained with DAPI nucleic acid stain, Figure 2D) and GFAP (red fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus, Figure 2E) positive immunoreactivities were found.

DISCUSSION

Since 1990s, many researchers have demonstrated that NSCs are widely present in central nervous system (CNS) including spinal cord[6,9-17]. NSCs not only have the probabilities of division, regeneration proliferation and multidirectional differentiation (differentiate into nearly all types of CNS nerve cells such as neuron and glial cell), but also exhibit low immunogenicity and good histocompatibility[6,9-17]. This refutes the theory that the damaged cells are unable to be regenerated and repaired following the injuries to the nerve and the spinal cord, and provides new hope to the reconstruction of structure and function in CNS disorders such as SCIs and cerebral injuries[6,18]. NSCs, as the seed cells enhancing CNS regeneration, spontaneously become cores and foundation of neurological research. Efficient approaches for enhancing pure NSC yield in the research thus becomes crucial. The present study established a method for NSC amplification, culture, purification and identification *in vitro* to provide sufficient-necessary condition for further investigation of NSCs. Cell culture technology is the basic premise for the investigation of NSCs. Mature techniques for the isolation and culture of NSCs have been developed. In addition to proving the presence of NSCs and its potential of self-renewal and multidirectional differentiation, Reynolds and Weiss *et al*[9] also demonstrated that NSCs could proliferate massively *in vitro*, which establish crucial conditions for future investigation of NSCs. However, application of methods for *in vitro* amplification of stem cells is currently restricted to CNS. By contrast, cloning of monolayer cells[6,19] offers an alternative approach for NSC culture. This

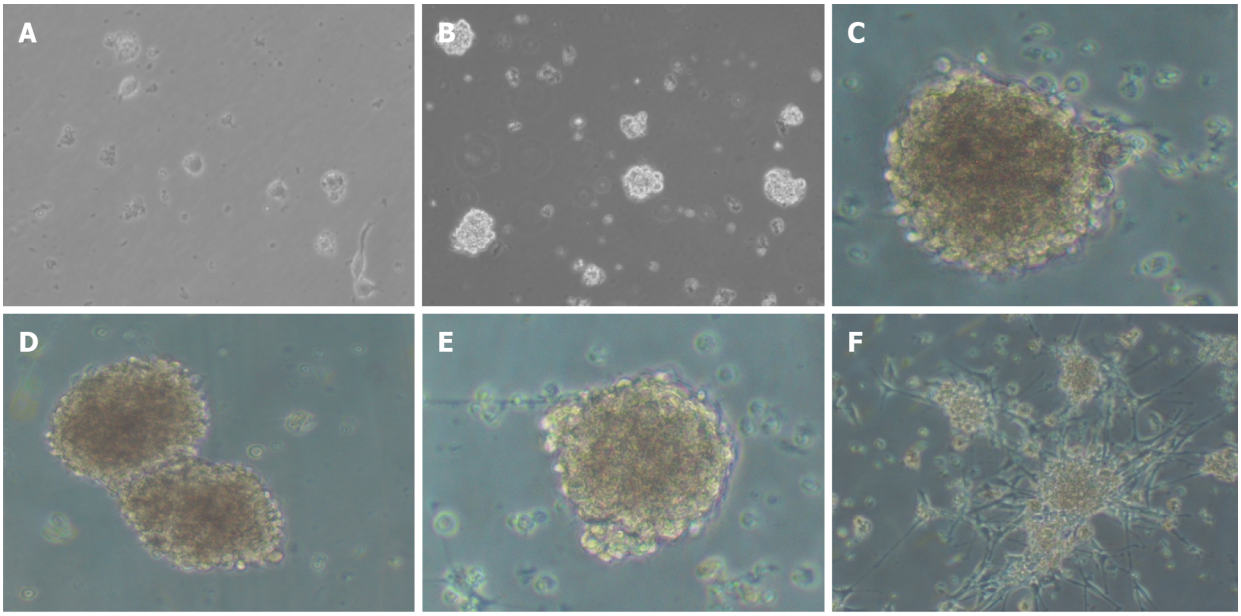

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Figure 1 Development of neural stem cells derived from newborn rats (inverted phase-contrast microscope). A: At 24 h after the primary culture (100×); B: 3 d after the primary culture (100×); C: 5 d after the primary culture (100×); D: 8 d after the primary culture (100×); E: 4 d after 5 passages (×200); F: 5 d after induction of dissociation (×200).

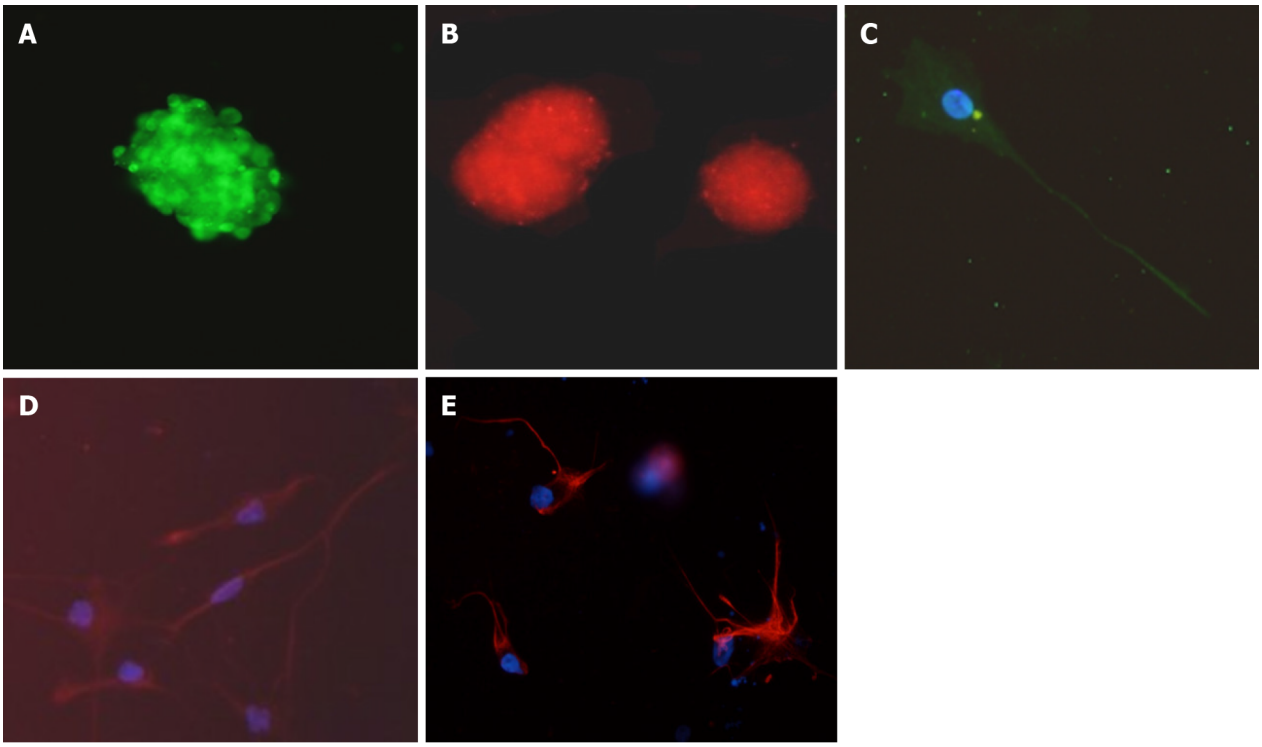

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Figure 2 Immunofluorescence staining. A: BrdU immunofluorescence staining (green fluorescence, ×100); B: Nestin immunofluorescence staining (red fluorescence, ×100); C: NF200 + DAPI immunofluorescence staining (green fluorescence refers to NF200, blue fluorescence refers to DAPI, ×200); D: NSE + DAPI immunofluorescence staining (green fluorescence refers to NSE, blue fluorescence refers to DAPI, ×200); E: GFAP + DAPI immunofluorescence staining (green fluorescence refers to GFAP, blue fluorescence refers to DAPI, ×200).

approach is widely used because using this approach we can not only derive an abundant amount of pure NSCs in a short time but also determine the differentiation capacity for a single NSC or its precursor cells.

For the timing of NSC passaging, the present study found that although passaging at two-to-three-day primary culture could facilitate the dissociation of neurospheres and formation of single-cell suspensions, the number of single cells derived was unsatisfactory at low densities which was not conductive to amplification and proliferation of NSCs after passage. Passaging performed when primary NSCs are cultured for seven to eight days could only dissociate neurospheres into small clusters of cells with a small amount of single floating cells instead of single-cell suspensions, which was presumably related to the tight junctions between peripheral neurosphere cells[20]. Moreover, small clusters of adherent cells with small portion of low density single floating cells were discovered after only two days of passage. Differentiation of adherent large neurospheres occurred after a continued cultivation. The observation of nine-to-ten-day primary NSC culture revealed that differentiation occurred in plenty of adherent large neurospheres with lots of dead cells, which also inhibits passaging. Comparatively, when passaging was performed after four to five days of primary culture, it not only promotes easy generation of single-cell suspensions dissociated from neurospheres but also harvests sufficient single floating cells with fewer dead cells and improved amplification and proliferation capabilities of NSCs. It follows that the optimal timing for NSC passage is after four-to-five-day primary or passage NSC culture. During the progress of *in vitro* culture, junctions can be formed between NSCs, which assemble to form neurospheres, through cytoskeletal protein and calnexin induced cell-cell adhesion mediation[20]. For neurospheres formed through primary culture or passage culture carried out for two to three days, the junction between NSCs is relatively loose making dissociation and formation for single floating cells easy. However, an analysis of the grow curve over this period displayed that passaging which is carried out when NSCs entered the logarithmic growth phase from the incubation phase might destroy the rapid amplification of NSCs resulting in passaged NSCs' repeated incubating and missing the optimal timing for amplification and at last failure to yielding sufficient NSCs in a short time. On the fourth to fifth day of primary culture or subculture, the number of NSCs increased significantly, the volume of neurospheres grew bigger and cell-cell junctions became tight when the junctions were still able to be dismantled by some means to obtain single cells comfortably. Notably, cell culture gradually reached a plateau period with anchoring cell-cell junctions over time. Oppositely, when the primary or passage culture last for seven to eight days, cell-cell junctions would become so tight that it was difficult to cleave or dissociate. Forced disconnection might end up with NSC damage, dissolution, death, adherence and differentiation. Furthermore, it was observed that the number of neurospheres decreased with their increasing volume suggesting the bulked volume of neurospheres might arise from neurosphere fusion instead of large amplification of NSCs. This can be interpreted as with the increasing volume of neurospheres, it is difficult for the cells within the spheres to contact the culture medium, which then prevents cells within the spheres from absorbing nutrition and affecting the process of metabolism, causing decline in the proliferation abilities of cells within the neurospheres, death of a large number of cells and generation of necrotic cores[21]. From this view, balance should be reached between easy dissociation of neurospheres into single cells and rapid amplification of NSCs without damaging the proliferation abilities of cells within spheres by the excessive volume of neurospheres or causing death during the choice of timing for NSC passage. After summarizing the results of experiments, it is believed that the optimal timing for NSC passage is on the fourth to fifth day of primary or passage NSC culture.

Currently, the commonly used methods for passaging are enzymatic digestion method and mechanical method. After analyzing the investigations[4,7,8], the present study discovered that: (1) Although mechanical method alone is easy to operate and needs a very few of centrifugation, the force and times of tapping and pipetting is difficult to control and it increases the probability of mechanical damage to cells, which is not conducive to the experimental operation; (2) for enzymatic digestion method, trypsin is mainly used. However, the effect of trypsin in digestion is likely to cause chemical damage to NSCs. Moreover, usage of serum or enzyme inhibitor to counteract the digestive effect of trypsin may reduce the vitality and proliferation rate of NSCs and then influence the experimental results; (3) concerning the combination of enzymatic digestion method and mechanical tapping and pipetting method, in spite of a very few of blasting is needed and small scale of blasting can dissociate neurospheres into single-cell suspensions, it does not elevate the number of single cells while the amplification capability of NSCs and number of neurospheres decrease significantly; and (4) in terms of TrypL™ Express digestion method combined with mechanical tapping and pipetting method, TrypL™ Express, a high purity recombinant enzyme, has trypsin-like property, which cleaves peptide bonds on the C-terminal sides of lysine and arginine. TrypL™ Express has high thermostability compared with trypsin. There is no need to use serum or enzyme inhibitor to compromise its digestive effect. Instead, the effect of TrypL™ Express is mild with less toxicity. However, the action of TrypL™ Express should not last long. This study used TrypL™ Express combined with mechanical tapping and pipetting methods as the passaging method. Using this passaging method, sufficient regular shaped, even sized sheen floating single cells were harvested. What's more, cell culture medium is clear with few fragments and elemental impurities, and NSCs holds vigorous growth and proliferation abilities. After observing the process of NSC culture post passaging, it discovered proper sized and good shaped colony-like neurospheres. Based on this, this study recommend using TrypL™ Express combined with mechanical tapping and pipetting method as the method for NSCs passaging.

Additionally, immunofluorescence staining was used to identify whether the cultured cells derived from newborn rats' brains were NSCs. The 5th generation of passaged cells showed positive after both BrdU immunofluorescence staining and nestin immunofluorescence staining. BrdU, a thymidine analog, can incorporate into newly synthesized DNA during the S-phase of the cell cycle[22]. Its property of continuous self-replication and self-reproduction by stem cells makes it take in labeled Brdu during replication. Thus, when immunocytochemical detection is carried out, BrdU positive suggests the cells are in the division and proliferation phrase. Accordingly, NSCs' potential of division and proliferation can be identified by Brdu detention. Nestin, also called NES, is a major cytoskeletal protein in stem cells in the mammalian CNS and its expression is inversely associated with cell differentiation[23-25]. Nestin is a specific biomarker for NSCs and functions as one of factors to identify NSCs. However, nestin is also expressed in pancreatic progenitor cells and endodermal cells. Therefore, nestin positive cells are not necessarily NSCs. The identification of NSCs cannot only based on a certain feature. Apart from nestin immunofluorescence staining, specific biomarkers for NSC differentiation should be considered. The present study selected neuron-specific biomarkers NF200 and NSE and astrocyte-specific biomarker GFAP respectively for the identification of muti-differentiation of NSCs. This study used immunofluorescence staining for the detection of cultured cells derived from newborn rat brains in respect of the above described some characteristics of NSCs. It was observed that the 5th generation of passaged culture cells showed BrdU positive after BrdU immunofluorescence staining and nestin positive after nestin immunofluorescence staining. NF-200, NSE, GFAP positive cells were discovered following immunofluorescence staining at five days after induction of dissociation was performed in 5% fetal bovine serum. This implied that the cultured cells fulfil the criteria for NSC identification - expression of nestin, abilities of differentiation, renewal and proliferation and moreover the capabilities of dissociation. The composition of all these factors can demonstrate the cultured cells are NSCs.

There still are limitations on the development of methodologies to efficiently isolate, culture, and identify NSCs *in vitro*, and evaluating these obtained NSCs is the precondition for its clinical application. Further studies should try to find relevant influencing factors behind these issues.

CONCLUSION

Above all, the approaches used in this study yields sufficient purified NSCs, which then are employed for the further study of NSCs.

ARTICLE HIGHLIGHTS

Research background

The present study explores to establish a simplified and efficient method for culture and identification of neural stem cells (NSCs) derived from newborn rats in the aspects of timing of passaging, passage number, passaging approaches and methods for cell identification.

Research motivation

The study of NSCs is crucial for it will provide important insight into understanding and treating brain disorders. Efficient way to harvest sufficient neural stem cells will facilitate the use of NSCs in clinical scenarios.

Research objectives

This study aimed to explore a more effective protocol for culturing and identifying NSCs derived from newborn rats.

Research methods

Passaging of brain tissues sections dissected from new born rats (2 to 3 d) was conducted using TrypL™ Express combined with mechanical tapping and pipetting techniques. After five passages, NSCs as well as the revived NSCs from cryopreservation were identified. BrdU incorporation method was used to detect self-renew and proliferation capabilities of cells. Different NSCs specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies) were used to identify NSCs specific surface markers and muti-differentiation capabilities with immunofluorescence staining.

Research results

With TrypL™ Express combined with mechanical tapping and pipetting techniques, spherical-shaped cell clusters were successfully obtained. When BrdU was incorporated into the 5th generation of passaged cells, positive BrdU cells and nestin cells were observed by immunofluorescence staining. After induction of dissociation using 5% fetal bovine serum, positive NF200, NSE and GFAP cells were

observed by immunofluorescence staining.

Research conclusions

The present study developed a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification.

Research perspectives

First, there still are limitations on the development of methodologies to efficiently isolate, culture, and identify NSCs *in vitro*. Second, evaluating these obtained NSCs is the precondition for its clinical application. Further studies should try to find relevant influencing factors behind these issues.

FOOTNOTES

Author contributions: Zhou QZ designed the study; all authors contributed to the data collect and manuscript writing; all authors have read and approve the final manuscript.

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