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Research article

A new method of isolating spinal motor neurons from fetal mouse

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HIGHLIGHTS

- A new method was described to isolate spinal motor neurons from fetal mouse.
- Single gradient of OptiPrep medium was demonstrated to effectively isolate motor neurons.
- Spinal motor neurons with high purity and good condition were successfully isolated.

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ABSTRACT

Background: Isolating of primary motor neurons from animal embryos is critical for the study of neurological disease including mechanistic discovery and therapeutic development. Density gradient centrifuge taking advantage of the buoyant of motor neuron permits the enrichment of motor neurons. Despite the metrizamide, an OptiPrep medium has been introduced to separate the motor neurons by gradient centrifuge.
New method: We hereby used single density gradient of OptiPrep medium to isolate the spinal motor neurons from the fetal mouse.
Results: Single density gradient of OptiPrep medium is effective to isolate spinal motor neurons from the fetal mouse. The immunofluorescence staining analysis showed that the purity of cultured motor neurons at 72 h was between 90% and 95%.
Comparison with existing method: Four gradients of OptiPrep medium have been previously used to isolate the motor neurons from spinal cord of mouse. In this study, the single gradient of OptiPrep medium was demonstrated to effectively isolate spinal motor neurons from the fetal mouse.
Conclusions: The single gradient of OptiPrep medium is enough to produce high purity of spinal motor neurons from the fetal mouse.

1. Introduction

Motor neurons characterized by large soma, long axons and wide-ranging dendritic arborization are responsible for voluntary movement. Dysfunction or injury of motor neurons are important contributors in the pathogenesis of multiple neurological diseases including amyotrophic lateral sclerosis, Kennedy's disease and spinal muscular atrophy (Graber and Harris, 2013a; Mandemakers, 2014). Isolation and culture of motor neurons facilitate our understanding and better strategies for these diseases. In this article, we described a new method of isolating primary motor neurons from the spinal cord of C57BL/6 mice.

2. Materials and methods

2.1. Animals

Female (ten–twelve weeks) and male (eight to ten weeks) C57BL/6 mice were purchased from Vital River Laboratories (Bei-

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jing, China) and housed at a controlled temperature in a 12 h light–12 h dark cycle with free access to food and water. The experiment was approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Jining Medical University and performed in accordance with the Principles of Laboratory Animal Care.

2.2. Reagents

Neurobasal medium, Hibernate-E medium, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12 basic), B-27 serum-free supplement (50×), L-glutamine (10 mg/mL), 0.25% Trypsin-EDTA, Trypan blue and penicillin-streptomycin were purchased from Gibco (NY, USA). Poly-L-lysine (MW 70,000–150,000), Dulbecco's phosphate-buffered saline (D-PBS) and Triton X-100 were purchased from Genview (TX, USA). OptiPrep™ density gradient medium and fetal bovine serum (FBS) were purchased from Sigma (MO, USA). Rabbit anti-Choline Acetyltransferase (ChAT) antibody, mouse anti-SMI32 antibody, Alexa-488-conjugated donkey anti-rabbit IgG, Alexa-568-conjugated goat anti-mouse IgG and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Abcam (MA, USA). Goat serum was purchased from Zhongshan Golden Bridge Company (Beijing, China).

2.3. Isolation of embryonic spinal cord

Male and female mice were mated at a ratio of 1:2. An embryonic date was identified by checking a vaginal plug and recorded as E 0 d. On E 13.5 d, the pregnant mouse was anesthetized with CO_2 and killed by cervical dislocation. After rapidly soaking in 70% alcohol for 30 s, the pregnant mouse was placed on a sterile 100-mm petri dish for the separation of embryos. All embryos were transferred into a new 100-mm petri dish containing 10 ml ice-cold D-PBS. The following steps were performed under a dissection microscope. Each embryo was quickly decapitated and the ventral side was placed down in the dish using toothed forceps. After the skin overlying the spinal cord was removed, the spinal cord was lifted out and placed into ice-cold PBS. The membrane of spinal cord together with the dorsal root ganglion was removed using microforceps. Then, the spinal cord was transferred into a new sterile 60-mm petri dish containing 0.5 ml ice-cold Hibernate-E for next step.

2.4. Mechanical and enzymatic digestion

The spinal cord was quickly cut into approximately 0.1 cm^3 slices using ultrafine microscissors and then transferred into 0.25% trypsin-EDTA solution which had been preheated at 37 °C. In a 37 °C, 5% CO2 humidified incubator, the spinal cord slices were digested for 25 min and triturated gently every 5 min using a sterile Pasteur pipette. The digestion was terminated by adding DMEM/F-12 medium supplemented with 2% FBS. The cell suspension was filtered using a 100 μm cell strainer (Boster, Wuhan, China) and then transferred into a 15-ml centrifuge tube for further separation.

2.5. Density gradient centrifugation

The cell suspension was centrifuged at 1000 rpm for 5 min at 4 °C with no brakes. The pellet was re-suspended in 4 ml of NABG medium (97% of Neurobasal medium, 2% of B27 serum-free supplement, 0.5% of 2 M L-glutamine and 0.5% of penicillin-streptomycin). To prepare the separation solution, OptiPrep medium was added into DMEM/F-12 medium to make a final concentration of 12.4% (v/v, for instance, 0.496 ml of OptiPrep medium mixed with 3.504 ml of DMEM/F-12 medium). 4 ml of separation solution was added into a new sterile 15 ml centrifuge tube. The cell suspension was gently layered onto the separation solution with a sterile Pasteur pipette. After centrifuging at 2200 rpm for 10 min at 4 °C, the cell suspension was separated into three layers. As shown in Fig. 1, the upper layer (F1) contains cell debris, and the motor neurons were concentrated in the second interface, the cloudy layer (F2). The final layer (F3) is the separation solution containing non-neural cells, e.g., capsule, fibroblasts and Schwann cells. The cloudy layer was carefully collected into a 15 ml centrifuge tube containing 3 ml of DMEM/F-12. After centrifuging at 1000 rpm for 5 min, the supernatant was discarded and the pellet containing motor neurons was gently re-suspended with an appropriate amount of NABG medium. Cell viability tested by trypan blue staining ranged from 95% to 98%.

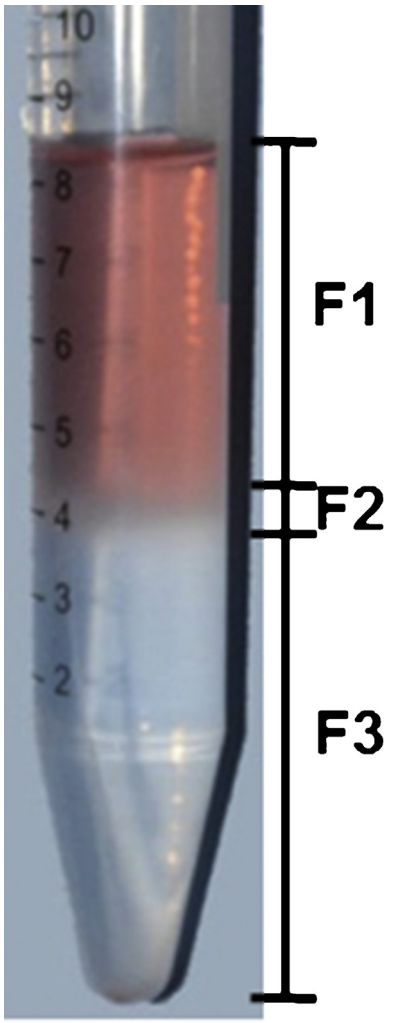

Fig. 1. Isolation of mice motor neurons by density gradient centrifugation using OptiPrep solution. As it was shown, 12.4% of OptiPrep solution separated the cell suspension of spinal cord into three layers, which were numbered as F1, F2 and F3. According to the principles of density gradient centrifugation, motor neurons were enriched in the F2 layer.

2.6. Culture and immunofluorescence

Re-suspended motor neurons were seeded at 1×10^4 cells/cm^2 on glass coverslips in a 12-well plate. The glass coverslips were pre-coated with 50 μg/mL poly-L-lysine for 2 h at 37 °C as was previously described (Ahlemeyer and Baumgart-Vogt, 2005). Motor neurons were cultured in a CO_2 (5%, 37 °C) incubator without disturbance. Half of the culture medium was replaced with fresh NABG medium every two days. The images of cultured motor neurons were captured at 0, 12, 24, 48 and 72 h respectively