Simple method to isolation and culture of neuron progenitor cells (NPCs) from whole brain post-natal rat

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Abstrak

Latar Belakang: *Neurobiologi dipelajari menggunakan sel neuron dari kultur primer atau menggunakan cell line tergantung pada tujuan penelitian yang akan dilakukan. Berbagai metode dikembangkan untuk mendapatkan sel neuron pada bagian korteks, hipokampus atau dari semua jaringan otak dari otak fetus atau tikus yang baru lahir. Sel neuron tidak mampu berproliferasi sehingga perlu dikembangkan isolasi neuron progenitor cells (NPCs). Penelitian ini bertujuan untuk mengembangkan metode isolasi NPCs dari jaringan utuh otak tikus yang baru lahir secara mudah dan praktis.*

Metode: *Jaringan otak diperoleh dari tikus Sprague Dawley umur 2 hari. Eksperimen dilakukan dalam dua tahap yaitu memasukkan jaringan otak dalam tripsin 0,05% diinkubasi selama 10 menit, menambahkan medium kultur, disaring dengan pori membran dan sentrifugasi selama 10 menit. Tahap selanjutnya membuang supernatan, tambahkan dengan HBSS-glukosa, dimasukkan ke dalam larutan Ficoll 35% dan 65% kemudian sentrifugasi, selanjutnya supernatan ditanam di cawan kemudian dipindahkan lagi pada cawan yang telah dilapisi dengan poly-D-lysine. Karakterisasi dilakukan dengan imunositokimia penanda neuron (NeuN dan microtubule-associated protein 2-MAP2) dan flow cytometry ($PSANCAM^+$ and $A2B5^-$).*

Hasil: *Dalam waktu kurang dari satu jam dengan menggunakan metode ini dapat diperoleh NPCs. Hasil menunjukkan bahwa diperoleh lebih dari 95% sel dengan $PSANCAM^+$ dan $A2B5^-$. Setelah dikultur selama 4 hari, sel positif terhadap NeuN and MAP2.*

Kesimpulan: *Telah berhasil dikembangkan metode isolasi NPCs dari jaringan utuh otak tikus baru lahir yang mudah dan praktis dengan viabilitas dan kemurnian tinggi.* ***(Health Science Journal of Indonesia 2018;9(2):63- 9)***

Kata kunci: *tikus baru lahir, neuron progenitor cells (NPCs), isolasi*

Abstract

Background: Neurobiology is studied by neuron cells from primary cultures or cell lines depending on the purpose of the research. Various methods were developed to obtain neuron cells in the cortex, hippocampus or from all brain tissue from the fetal brain or newborn mice. Neuron cells are unable to proliferate therefore the isolation of neuron progenitor cells (NPCs) needs to be developed. This study aims to develop a method of isolating NPCs from intact tissue of newborn mouse brains easily and practically.

Methods: Brain tissue was obtained from Sprague Dawley rats aged 2 days. Experiments were carried out in two stages which included add trypsin 0,05% to brain tissue and then incubated for 10 minutes, adding culture medium, then filtered with pore size membrane and centrifuging for 10 minutes. The next step is to remove the supernatant then add with HBSS-glucose, put it in a 35% and 65% Ficoll solution and centrifugation, then the supernatant is planted in dish and then transferred again to the dish with poly-D-lysine cup. Characterization of neuron marker was carried out by immunocytochemistry (NeuN and microtubule-associated protein 2-MAP2) and flow cytometry ($PSANCAM^+$ and $A2B5^-$).

Results: In this study, our result show that this method does not take longer than one hours and more than 95% cells that obtained are expressing $PSANCAM^+$ and $A2B5^-$. After 4 days culture, cells exhibit positive for neuron marker as MAP2 and NeuN.

Conclusion: Successfully developed the easy and practical method to isolate NPCs from the whole brain of post-natal rat with high viability and purity. ***(Health Science Journal of Indonesia 2018;9(2):63-9)***

Keyword: post-natal rat, neuron progenitor cells (NPCs), isolation

Neuron cells are needed for in vitro neurobiology studies such as neurotoxicology studies, molecular signaling pathway and pathogenesis studies of neurodegenerative.[1,2] Neuron cell can be obtained from a primary neuron or neuronal cell lines, depend on the aim of the study because both are not equivalent. The limited proliferation capacity of primary neurons necessitates further study to optimize neurons isolation techniques.

In developing and adult mammalian brain, there are NPCs that maintain the regeneration process of neuronal cells. NPCs are multipotent cells committed to the neural lineage that can self-renew, proliferate and differentiate so it can be expanded in vitro.[3,4] NPCs are normally quiescent and reside within specific niche including subventricular zone (SVZ) of the lateral ventricle, the subgranular zone of the dentate gyrus (DG) of the hippocampus and periventricular region surrounding the central canal of spinal cord.[5,6]

Various methods are performed to obtain NPCs from cortical, hippocampal and whole brain of adult, neonatal and prenatal rat.[5,7–9] Each method has some effectiveness and advantages which is different. Isolation NPCs from specific region need high skill, the dissecting tool and stereomicroscope to cut SVZ and DG area of the one adult rat brain.[5,9] This study aimed to develop an easy and practical protocol for NPCs isolation.

METHODS

The experiment was conducted in stem cell and animal laboratory of Center for Research and Development of Biomedical and Basic Technology of Health (CRDBBTH), National Institute of Health Research and Development (NIHRD), Ministry of Health of Indonesia from March to October 2016. All ethical clearance was approved by The Ethical Committee of NIHRD.

Isolation. Three Sprague Dawley (*Rattus novergicus* L) age 1-2 days old rats were used in this study. The termination was injected intramuscularly by Ketalar. The postnatal rats are decontaminated with 10% iodine for 1 minute. Rinse 3 times with PBS before taking the brain. Using scissors and tweezers, peel away the skin and lift up the skull to expose the brain (do in the biosafety cabinet/BSC). Remove the whole brain into the petridish with 1% Hank Balanced Salt Solution (HBBS)-glucose then weighed with the sterile chamber. Taking the brain into the 15 mL of the tube with 1 mL of 0,05% trypsin-ethylenediaminetetraacetic acid (EDTA) for 400 g brain and incubated in the 37^0C dan 5% CO2 for 10 minutes. Adding culture medium with trypsin-EDTA comparison 1:1 and do the tirturation. Adding HBSS-glucose 5 times the volume of trypsin. Filter with 70 µm pore size membrane and then centrifuge 2000 rpm for 10 minutes. Remove of supernatant (fraction 5) and add 1 mL of HBSS-glucose (A).

Sorting. Preparing Ficoll dilution was done at one day before dissection.

- Reagents that need to be prepared : Ficoll (pH± 7,4 , 8 mL 2% NaHCO3 stock, CMF-PBS-EDTA (calcium and magnesium free-phosphate buffered saline-EDTA): per liter add 32 g NaCl, 1.2 g KCl, 8 g Glucose, 2 g NaH2PO4, 1 g KH2PO4, 8 mL 2% NaHCO3, 10 mL 1M EDTA to 1 liter deionized water. Adjust the pH 7,4 and filter sterilize.
- Preparing 35% and 60% Ficoll on the day of isolation, the composition:

Reagents	mL	
	Ficoll 35%	Ficoll 60%
Ficoll	3,5	60
CMF-PBS-EDTA	2,5	2,5
Milli-q water	4	1,5
Toluidine Blue	-	0,001
Total	10	10

Preparing Ficoll gradient; 5 mL 35% Ficoll in the 15 tubes, add 5 mL 60% Ficoll at the bottom of the tube with 10 mL pipette volume with slowly and add 1 mL of cells suspension (A), carefully insert the tube into the centrifuge and centrifuged at 1800xg for 10 minutes. Remove fraction 2,4, 5 and add 10 mL HBSS-glucose, then centrifuge at 1100xg for 5 minutes. Remove the supernatant and resuspended pellet with 1 mL culture medium.

The next step of sorting is pre-plating the cells suspension in the 35 mm (2 pcs) dish with poly-D-lysine 100 µg/mL coated pre-plating dish for 10 minutes in a 5% CO2 37°C repeat 2 times. Coated poly-D-lysine 100 µg/mL for 24 hrs before used and rinse with PBS 2 times. Taking the cells suspension and centrifuged 200xg for 5 minutes. Add 6 mL culture medium with cells resuspended, counting cells, seeding at 12 well @500 µl and characterized with flow cytometry.

Culture. Culture medium supplemented with fetal bovine serum 5% (FBS, Sigma), supplement N2 1% (Sigma) dan gentamicin 50 µg/mL (Sigma). The cells suspension were incubated at 37^0C and 5% CO_2. The medium replacement was done every two days. After 4 days of culture, cells were characterization with immunocytochemistry.

Immunocytochemistry. After 4 days, cells culture characterized with MAP2 and NeuN. The medium was discarded from well then washed with PBS twice. Fixation was done by 4% paraformaldehyde (PFA) for 15 minutes then washed with PBS 3x each for 5 minutes. Blocking steps are consist of (i) blocking of endogenous peroxidase with 3% H_2O_2 in methanol (Merck K38122297) for 15 minutes and (ii) blocking of nonspecific background staining with background snipper (Starr Trek Universal HRP Detection Kit Biocare®) for 15 minutes then washed in PBS 3x each for 5 minutes. Overnight incubation with microtubule-associated protein 2-MAP2 (Santa Cruz sc-74421) and NeuN (Abcam ab104225) at 4ºC then washed in PBS 3x each for 5 minutes. Further incubation with the secondary HRP-conjugated antibody (Trekkie Universal Link, Starr Trek Universal HRP Detection Kit Biocare®) for15 minutes then washed in PBS for 5 minutes. Followed by incubation with Trek-Avidin-HRP (Starr Trek Universal HRP Detection Kit Biocare®) for 15 minutes then washed in PBS for 5 minutes. Incubation in chromogen substrate diaminobenzidine (DAB) added with substrate buffer (Starr Trek Universal HRP Detection Kit Biocare®) for 1-2 minutes and washed with *milli-q water* for 10 minutes. Counterstain with *Hematoxylin Mayer* (Biocare3570) for 1-2 minutes. The final wash in *milli-q water* for 5 minutes. Positive and negative control was included in every staining protocol.

Characterization with a flow cytometer. Neuron progenitor cells are characterized using $A2B5^-$-APC and $PSANCAM^+$-PE-A (Miltenyi Biotec) markers with flow cytometer at 0-day culture. The use of staining methods is done according to instruction kit.

RESULTS

In this methods were used scissors and tweezers to lift the whole brain. It was done after the decontamination process in the BSC. Scissors are used to cutting the skin and skull while tweezers are used to removing dura meter and whole brain (Figure 1).

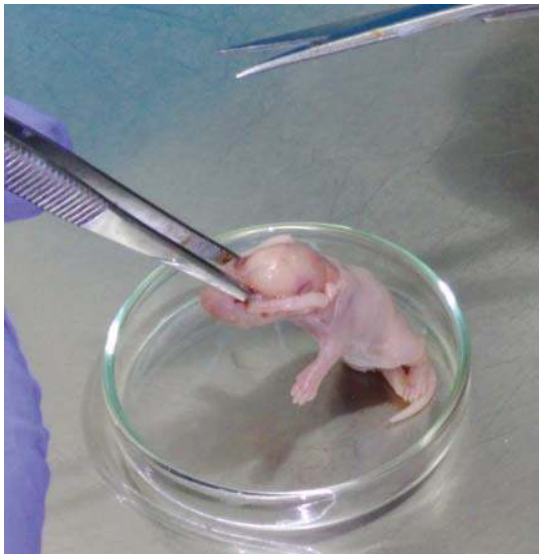

Figure 1. Lifting the whole brain from the post-natal rat.

This study are using 2 principles of the methods. First, the cells isolation were performed using enzymatic digestion and second, gradient centrifugation. It was used 35% and 65% concentration of Ficoll and 1 mL of cells suspension, then centrifuge at 1800 g for 10 minutes. After centrifugation, discard the supernatant and the pellet (fraction 5) suspended by culture medium to terminate the digestion (Figure 2).

Cell suspension filtered with a nylon mesh (pore 70 µm) before pre-plated in the plate that has been coated by poly-D-lysine. Pre-platting was done twice to get NPCs, in the 0 day, the morphology of NPCs still round shape (Figure 3A). After 4 days culture, NPCs morphology already bipolar (Figure 3B).

Characterization was done after 4 days culture in 37^oC and 5% CO_2. Immunocytochemistry staining with primary antibody rabbit anti-rat polyclonal MAP2 and NeuN (1: 50) was added to coloring the existing protein marker in the nuclei (NeuN) and the cytoplasm (MAP2).

Characterization also was done by identifying specific surface protein expression of NPCs by flow cytometry. Specific marker for verification of NPCs derived from whole brain post-natal rat were $PSANCAM^+$ and $A2B5^-$. At 0-day culture, verification was done. After 4 days culture, the expression of $PSANCAM^+$ and $A2B5^-$ decreased (data not showed).

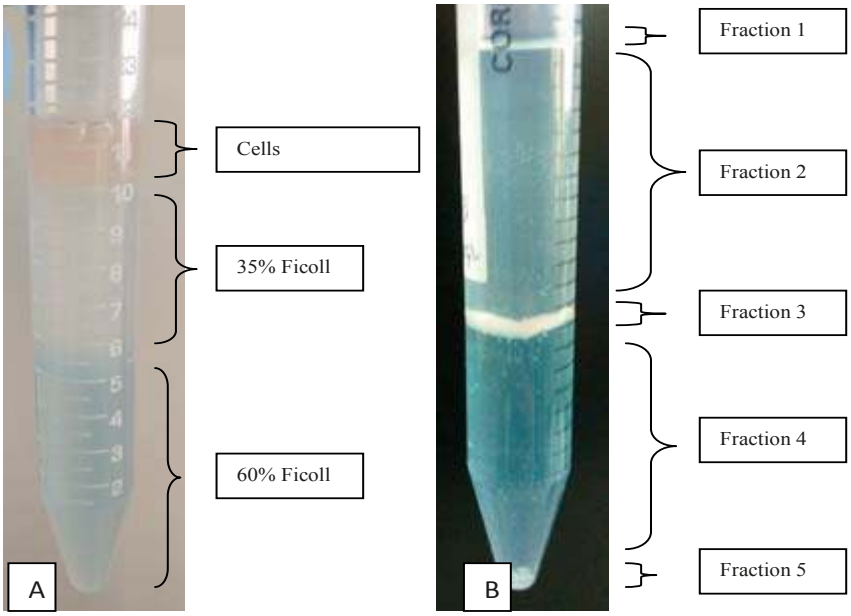

Figure 2. Ficoll Gradient. Fraction 5

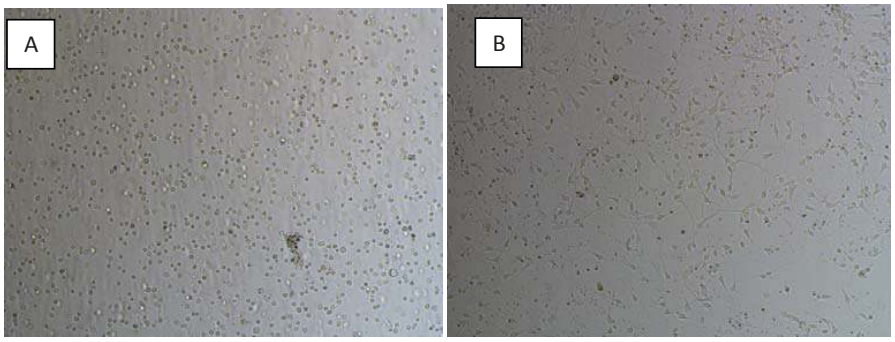

Figure 3. Morphology of neuron progenitor cells (NPCs) after 4 days culture. 40x

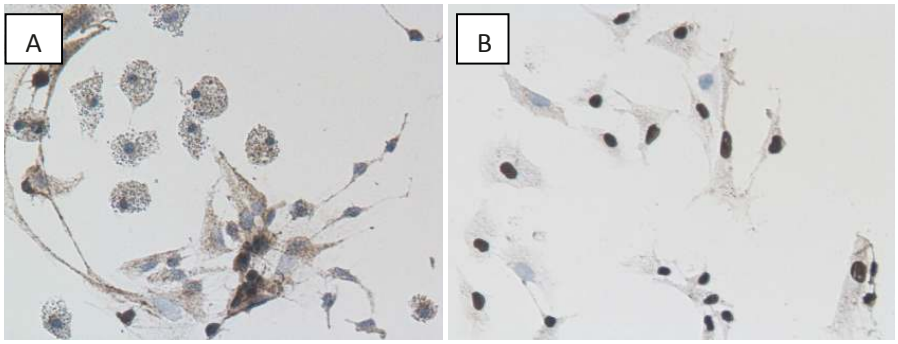

Figure 4. Characterization of NPCs with positive marker MAP2 (A) and NeuN (B) after 4 days culture. Morphology NPCs after immunocytochemistry staining (400x).

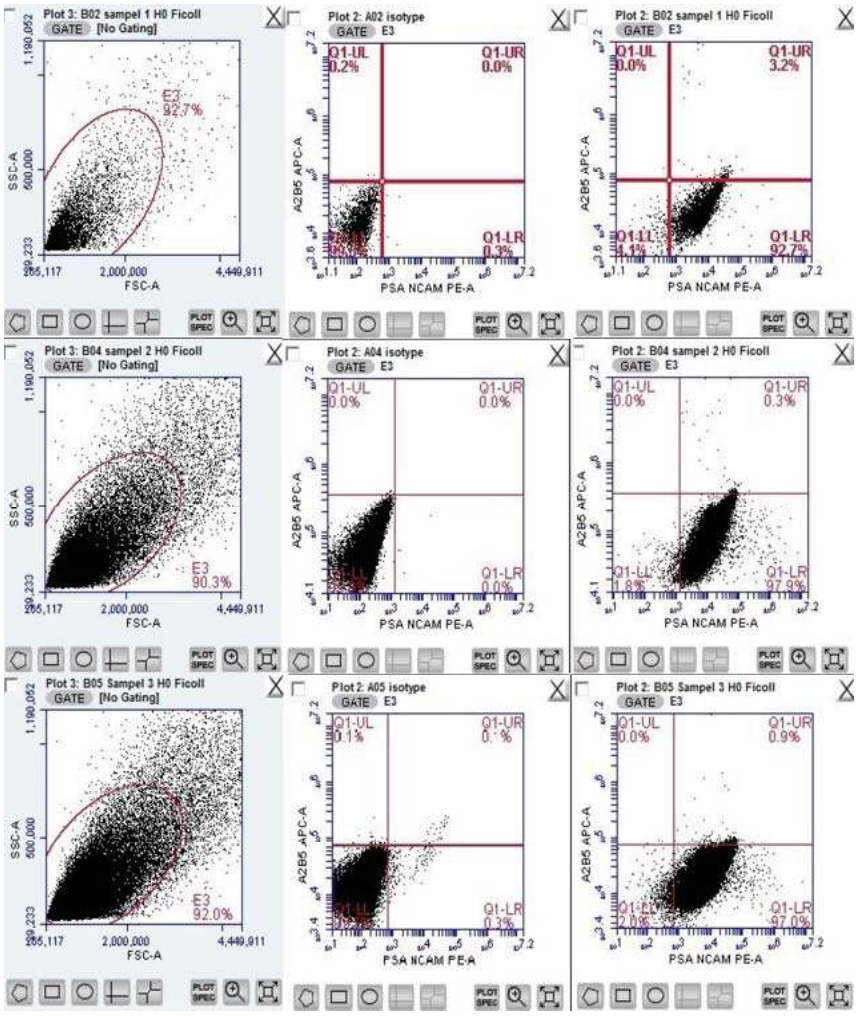

Figure 5. Flow cytometry analyzes the purity of PSA NCAM^{+} dan A2B5^{-} after isolation with Ficoll gradation was 95,86% at day 0.

DISCUSSION

This protocol based on a modification of procedure that have been reported by Xu et. al. (2012) dan Lee et. al. (2009) that isolated over 95% of the neuron (β-tubulin marker).[7,9] Tools that used isolation the cortex more complicated than the whole brain such as scissors, microsurgical tweezers, and stereomicroscope. In this protocol, we just need two tweezers and one scissor. Isolation was done at BSC to minimize contamination when culture. We got 97% purity of NPCs with this method (Figure 5). This protocol more easy, simple and effective because not need expensive tools so can be done by the laboratory with minimum equipment.

The enzyme used for dissociation brain tissue in this protocol is trypsin. This enzyme is quite easy to get and is cheaper than another enzyme that used by other protocol such as papain, DNase or dissociation kit. Selection of enzyme affects the time of dissociation step. Using with papain or DNase 1 take 20-30 minutes.[9] The dissociation step in this study was shorter in duration than previous studies.

This step very influences the cells viability that will be collected. The cells viability also affected by termination after the digestion process. In this protocol culture medium (5% FBS) used to terminate the digestion but Xu et. al. use 1 mL FBS to terminate.[9] Although this study used fewer concentrations of FBS but the effectiveness of stopping the work of enzyme did not decrease. It showed that after dissociation the viability and morphology of NPCs were still good (Figure 3).

For increasing and purifying the yield of progenitor neuron were be replated twice with poly-D-lysine

(P-DL) (100µg/mL) for 10 minutes, in this study. The repetition was done to separate astrocytes, heavier cells, and NPCs. Astrocytes and heavier cells will settle down and adherent to the dish faster than NPCs. Lee et. al. (2009) and Xu et. al. (2012) were managed to pre-plating with coating dish with poly-D-lysine and L-polylysine (L-PLL) for 20 minutes.[7,9] Extracellular matrix (ECM) e.g P-DL and L-PLL provides support to enhance NPCs expansion and differentiation.[10–12] They also function as adhesive substance so NPCs easily attached and detached; for further replating we only using pipetting up and down to agitate the cells.

After isolation, the cells viability, proliferation and differentiation ability of NPCs depend on the culture medium, temperature, pH and gas. Culture medium contains various growth factor are nutrient which is needed for proliferation and differentiation by NPCs. Xu et al. and Lee et. al. used neurobasal medium supplemented with B27, 10% FBS and GlutaMAX for culturing NPCs.[7,9] In this protocol we used neurobasal medium supplemented with N2 and 5 % FBS. In the FBS, there are a lot of growth factor such neurotrophic factor that can maintain NPCs. Supplements such as B27 and N2 are consists of glycoprotein and chemical to support the metabolism and molecular signaling of NPCs.[13,14] Its showed by validation the purity of cell that collected are more than 95% positive with PSANCAM marker on day 0.

In this research, characterization of NPCs was done with antigenic markers such as PSANCAM, A2B5, Neun, and MAP2. NPCs isolation using $PSANCAM^+$ and $A2B5^-$ based on Seidenfaden and Azari research. Seidenfaden et. al. report that there are 2 subpopulations of cells from isolated of post-natal mice brain. There are the $A2B5^+$ fraction contains enriched glial progenitors and the $A2B5^-/PSANCAM^+$ fraction that contains committed neuronal precursors.[15] Azari et. al. report that isolation of immature neuronal cells from the adult mouse subventricular zone (SVZ) can using marker $PSANCAM^+$ and using marker $A2B5^-$ to increase pure population of immature neuron population as marker of immature glial cells.[16]

In conclusion, it's successfully developed the easy and practical method to isolate NPCs from the whole brain of post-natal rat with high viability and purity.

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