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THE CONDITIONED MEDIUM-RAT BONE MARROW DERIVED MESSENCYHMAL STEM CELL (CM-RATBMMSC) CAN INDUCE THE DIFFERENTIATION ABILITY OF NEURAL STEM AND PROGENITOR CELLS (NPCS)

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Key word *: Conditioned medium /CM, mesenchymal stem cell/MSC, Rat bone marrow derived MSCs, Neural stem and progenitor cells/NPCs*

Abstract– Mesenchymal stem cell (MSC) and conditioned medium-MSC (CM-MSC) are potential therapeutic agents for the treatment of neurogenerative diseases. Conditioned medium-mesenchymal stem cell (CM-MSC) contained growth factors that can protect neuronal cells from cell death and induce differentiation of neural stem/progenitor cells (NPCs). **Aims** : To investigate the capability of conditioned medium rat bone marrow-derived mesenchymal stem cells (CM-rat BMMSC) induces in vitro differentiation of NPCs into astrocytes and neuron. Methods : We assessed the effects of CM-ratBMMSC to induce differentiation of NPCs by culturing cells in serum-free medium DMEM/F12 (FM), CM 100%, CM 50% and neuro basal medium with supplement (NM) for 4 days. We examined the effects on differentiation by assessing the expression of A2B5, PSANCAM, and beta-tubulin by flowcytometry, and the expressions of glial fibrilary acidicprotein (GFAP) and neuronal nuclei (NeuN) by immunocytochemistry (ICC). **Results**: CM-ratBMMSC induced differentiation of NPC to astrocytes and neurons which indicated by the expression of GFAP, beta-tubulin and NeuN. Our findings showed that growth factors or neurotrophic factors such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) in CM-ratBMMSC induces NPCs differentiations that may happen via phosphoinositide 3-kinases (PI3K)/protein or kinase B (Akt) signalling pathway. **Conclusions** : Growth and neurotrophic factors from CM-rat BMMSC such as bFGF and NGF increased the differentiation ability of NPCs into astrocytes (GFAP) and neurons (NeuN).

INTRODUCTION

Mesenchymal stem cell (MSC) is multipotent adult stem cells and plastic adherent stromal cells which can differentiate into bone, cartilage and adipose tissue. (Tondreau *et al*., 2004) It can be obtained from dental pulp, bone marrow and wharthon's jelly. (Inoue *et al*., 2013)',(Tondreau *et al*., 2004; Zhang *et al*., 2017) Mesenchymal stem cell (MSC) has a promising prospect as a regenerative medicine for neurodegerative diseases. These cells can transdifferentiate into mesodermal lineage such as neural lineage (Ahmedy *et al*., 2015). Moreover, previous studies reported that MSC has an ability to differentiate into neuronal cells and also can secrete growth factors or cytokines for neurological function improvement. (Tondreau *et al*., 2004; Ahmedy *et al*., 2015; Kurozumi *et al*., 2005) MSC secretes neurotrophic and anti inflammation factor so that it reduced ischemic damage in stroke and spinal injuriy (SCI) in animal model. (Kurozumi *et al*., 2005); (Cantinieaux *et al*., 2013) Currently, it is believed that not only the cells can be used as therapeutic agents but also the conditioned medium (CM) derived from MSC. (Pawitan, 2014)

Conditioned medium (CM) is a medium from specific cells culture such as stem cell culture that contained factors/molecules secreted by the cells to

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the extracellular space. (Vizoso *et al*., 2017; Pawitan 2014) Conditioned medium contains soluble factors, such as cytokines interleukin 10 (IL10) and tumor necrosis factor (TNF); growth factors such nerve growth factor (NGF), brain derived nerve factor (BDNF), insulin growth factor (IGF); and basic-fibroblast growth factor (bFGF) which plays a role for neuroprotection and neurogenesis. (Woodbury and Ikezu, 2014; Nakajima *et al*., 2017); (Nguyen *et al*., 2010); (Sun *et al*., 2018). The advantages of CM application compared to the cells itself are it is safe, it can be mass produced, and it is more easy to transport and package (Pawitan, 2014)

The preclinical studies of MSC and CM-MSC for neurodegenerative diseases such as stroke and SCI in animal model had been successfully done, but it is important to explore various factors that promote and initiate neuronal cell regeneration and prove whether CM MSC can maintain and induce NPCs differentiation or not. Because of these reasons, we did in vitro studies to investigate whether CM–rat BMMSC and its growth factor contents can maintain and induce differentiation of NPCs into neuronal cells.

METHODS

Isolation and Culture of rat BMMSC

Wistar rats (12-week-old males) were used. Bone marrow was collected from femurs and tibias. Bone marrow was extruded by inserting a 22-gauge needle into the shaft of the bone and flushed out with 1 mL complete culture medium consist of DMEM/F12+ glutamax (Gibco), 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Gibco 15,240,062, Carlsbad, USA) incubated at 37°C with 5% CO_2. At 24 h after initial plating, the cells were washed twice with phosphate-buffered saline (PBS) to remove non adherent cells. The next day, medium must be replaced and every 2 days the culture medium was changed for 7 days. First passage was done with 0,05% trypsine EDTA (Gibco) and cells replated ($6x10^5$cells/mL) in 25TC flask.

Collection the CM-rat BMMSC

Conditioned Medium–rat BMMSC collected from 2nd passage of rat BMMC. After 2 days culture with complete culture medium, the flasks were washed twice with PBS and the medium was changed with 2 ml serum-free culture medium (FM; consisting of DMEM/F12 + glutamax and 1% antibiotic-antimycotic) for 24 hours. CM was collected and centrifuged at 1500 rpm for 5 minutes continued at 3000 rpm for 3 minutes. The CM was filtered with 0,22µm size of pore and further used for NPCs culture.

Isolation and Culture of Neural Stem and Progenitor Cells (NPCs)

NPCs were isolated from Wistar rat embryos on embryonic day 17 (E17). Pregnant female Wistar rat was euthanized by intraperitoneal injection of ketamine xylazine cocktail (91 mg/kg ketamine + 9.1 mg/kg xylazine) 0.1mL/100 g body weight. Lower abdomen was sprayed with 70% alcohol. The uterus was exposed by medial cutting through the skin. All fetuses were removed from the uterus then placed in sterile dissection solution (HBSS containing 0.3% glucose). Whole brains were isolated from the foetuses and dissected into small pieces then rinsed 3 times with dissection medium. Dissected tissues were centrifuged at 300 x g for 2 minutes then supernatant was discarded. Digestion process of brain tissues to obtain the cells were done using Neural Tissue Dissociation Kit (T) (Miltenyi Biotec). All the procedures were based on manufacturer protocols. Resuspended cells were seeded in coated dishes (1% poly D-lysine (PDL) and 1% gelatin for 24 hours) at cell density of 5×10^4 cells/cm^2in 24 wells plates. After 4 days culture, cells were characterized by immunohistochemistry and flowcytometry.

The medium used in this study was neurobasal medium (NM) consisted ofMACS® neuro medium (Miltenyi Biotec) containing 2% MACS NeuroBrew-21(Miltenyi Biotec), 1% antibiotic antimycotics (100x) (GibcoTM) and 1% GlutaMax® (GibcoTM). Conditioned medium 100% (CM100%) was CM-rat BMMSC only, while CM50% was CM-rat BMMSC and NM (1:1).

Flowcytometry

In this study, the rat BMMSCs were characterized with $CD29^+$-FITC,$CD90^+$-APC and $CD34^-$-PE (Biolegend). The NPCs were characterized using $A2B5^-$-APC, $PSANCAM^+$ -PE-A (Miltenyi Biotec) and beta tubulin-FITC (Biolegend) markers by flowcytometry at fourth day of culture. Staining process was done according to the instruction of staining kit. Flowcytometry process and analysis were done using BD Accuri™ C6 Plus flowcytometer.

Immunocytochemistry

Cells were characterized after 4 days of culture by immunocytochemistry with GFAP and NeuN markers. Medium was discarded from the wells then, the cells were washed twice with PBS. Fixation of the cells was done with 4% paraformaldehyde (PFA) for 15 minutes then were washed with PBS three times for 5 minutes each continued with blocking steps. Blocking steps consisted of (i) blocking of endogenous peroxidase with 3% H_2O_2 in methanol (Merck K38122297) for 15 minutes, (ii) blocking of nonspecific background staining with background snipper (Starr Trek Universal HRP Detection Kit Biocare®) for 15 minutes. After blocking steps, samples were washed in PBS 3x for 5 minutes each then were incubated overnight with primary antibody, GFAP (Santa Cruz sc) and NeuN (Abcam ab104225) at 4ºC. For the next process, samples were washed in PBS 3x for 5 minutes each and further incubated with secondary HRP-conjugated antibody (Trekkie Universal Link, Starr Trek Universal HRP Detection Kit Biocare®) for 15 minutes and were washed in PBS for 5 minutes followed by incubation with Trek-Avidin-HRP (Starr Trek Universal HRP Detection Kit Biocare®)for 15 minutes. Samples then were washed in PBS for 5 minutes and incubated in chromogen substrate diamino benzidine (DAB) with the addition of substrate buffer (Starr Trek Universal HRP Detection Kit Biocare®) for 1-2 minutes and were washed with mili-q water for 10 minutes. Counterstaining was done with Hematoxylin Mayer (Biocare3570) for 1-2 minutes and final steps were washed the samples with miliq water for 5 minutes. Positive and negative control was included in every staining protocol.

ELISA for CM-rat BMMSC

The concentration of bFGF dan NGF from CM-rat BM MSC were measured by Elabscience ELISA kitand inspection method adjusted according to the manufacturer's instructions.

Statistic

Data expressed as means±SD. P values were calculated using one-way ANOVA analysis.

RESULTS

The characteristics rat BMMSCs were analyzed by flowcytometry to determine the expression of cell surface markers. The results showed highly (e" 70%) expressed positive marker MSC (CD90 and CD29) and less than 1% of CD34 (haematopoietic specific marker) in the 2nd passage (Figure 1).

The characteristics of CM-rat BMMSC were

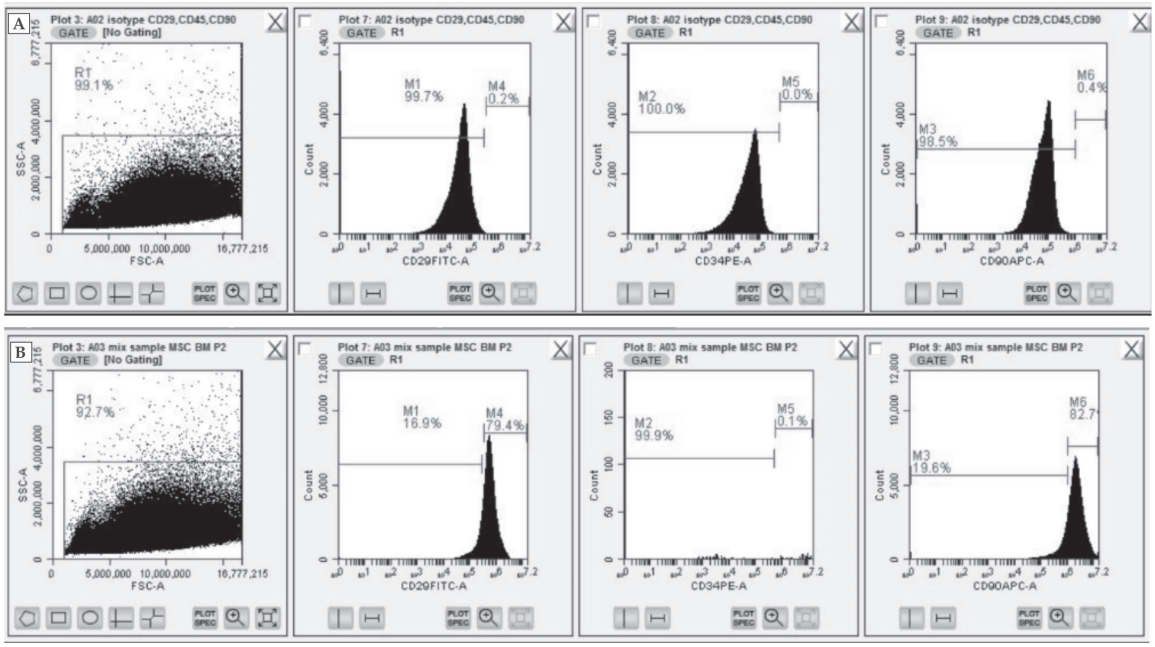

Fig. 1. Flow cytometry of $CD29^+CD90^+CD34^-$ rat BMMSCs after 5 days culture at passage 2 in which their conditioned medium (CM) will be collected and utilized to induce NPCA. Isotype of CD29, CD90 and CD34 < 0,5%. B. Percentage of CD29 and CD90 > 70% .

analyzed by ELISA to determine the secretion of growth factors such as bFGF and NGF. The result showed that CM100 from the 2nd passage of rat BMMSC contained both of growth factor as shown in Table 1.

Table 1. Concentration of bFGF and NGF of CM-rat BMMSC

Sample	bFGF (pg/mL)	NGF (pg/mL)
FM	0	0
CM100	625,823	16,83
NM	0	12,5

Characteristics of NPCs were analyzed by flowcytometry to determine the expression of cell surface markers after 4 days of culture with CM50 and NM with different extracellular matrix. There were less than 50% double expression of PSANCAM and beta-tubulin while the expression of PSANCAM were more than 50%when the cells cultured in CM50 on plates coated with PDL (Figure 2). However, culturing cells in NM on plates coated with gelatin showed that double expression of PSANCAM and beta-tubulin were less than 50% as similar as the percentage of the expression of PSANCAM only (Figure 3).

Characterizations by immunocytochemistry with GFAP and Neu-N markers were done after 4 days culture in 37°C and 5% CO_2. Cells that grew in CM100 were qualitatively positive for NeuN markers as shown in Figure 4.

Quantification of positive expressions of NeuN and GFAP markers showed high percentage in all groups (FM, CM100, and NM) with significant differences between free-serum medium and NM or CM100 medium (Table 2).

Table 2. Percentage of NeuN and GFAP of NPCs

Medium –*coating*	% NeuN	% GFAP
FM– PDL	43.88 ±2.5*a,b,c	28,66 ±2.7*a,b,c
NM – PDL	63.28 ±2.57*a,b	67.79 ±3.84*a,b
CM100 - PDL	61.16 ±4.11*a,c	68.77 ±4.37*a,c

* a, b,c Values with different letters within a column indicates significant differences

DISCUSSION

Isolation and culture method for rat BMMSC in this research were very simple but can produce high purity of MSC (Figure 1) eventhough separation method would increase positive surface markers of

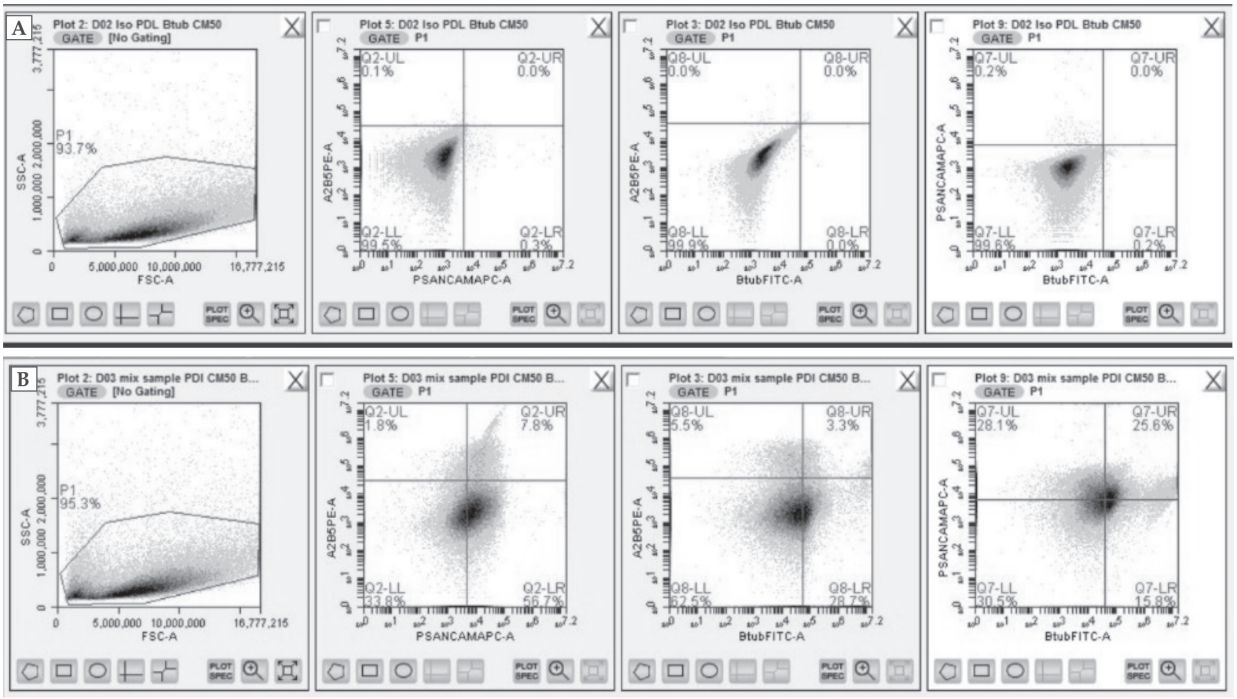

Fig. 2. CM-RatBMMSCs affected the cell differentiation to be predominantly a PSANCAM and Beta Tubulin (marker for neuron) after 4 days culture of NPC with CM50 and poly-D-lysine as extracellular matrix . A. Isotype of A2B5, PSANCAM and beta Tubulin < 0,5%. B. The purity of PSA $NCAM^+$ and Beta $tubulin^+$ > 20% and $A2B5^-$ <10%.

MSC (CD73, CD105, CD90, CD29). (Zhang and Chan 2010) Simple technique to separate MSC and haematopoeitic stem cells (HSC) by washing with PBS after 24h plating were effective to eleminate HSC that could not attach to the petridish. This method was done by Li *et al.,* (2013) who reported direct adherent was better than density gradient sentrifugation (Li *et al*., 2013) Isolation technique will affect the purity of MSC culture in which expected has a correlation with optimal growth factor secretions in CM. In the other hand, suplement in culture medium was important to increase MSC proliferation as reported by previous study that addition 11% FBS for culturing of rat BMMSC was better than 10% FBS. (Li *et al*., 2013)

Conditioned medium (CM)-MSC that contained some growth factors were potential for inducing differentiation into some types of cell from

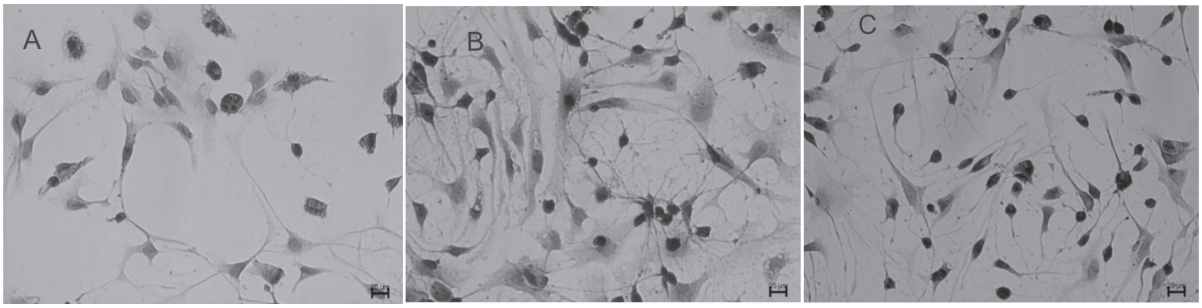

Fig. 4. Growth factors in CM100 induces neurogenesis *in vitro*. Immunocytochemistry of NPC showed positif expression of NeuN (marker for neuron) after 4 days culture. (A) serum-free culture medium (DMEM/F12+glutamax), (B) neurobasal medium (NM), (C) CM100. Scale bar = 25μm.

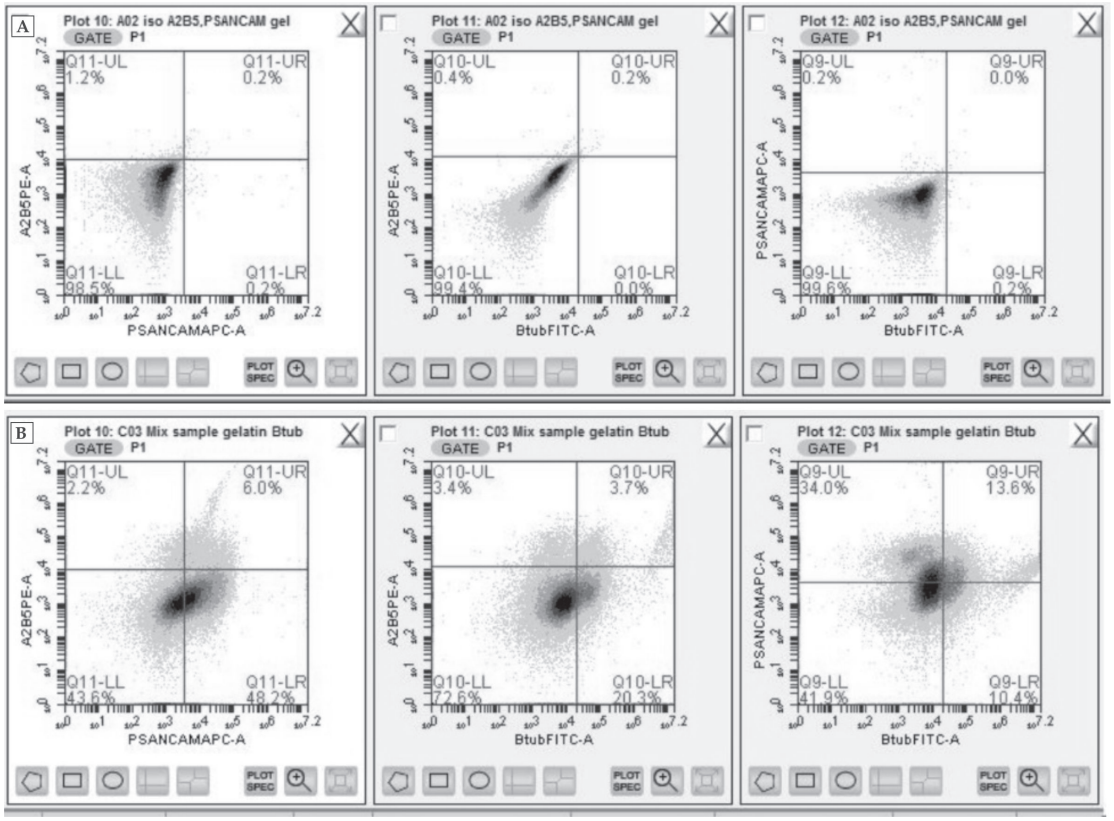

Fig. 3. CM-RatBMMSCs affected the cell differentiation to be predominantly a PSANCAM and Beta Tubulin (marker for neuron) after 4 days culture of NPC with neurobasal medium and gelatin as extracellular matrix. A. Isotype of A2B5, PSANCAM and Beta tubulin < 0,5%. B. The purity of PSA $NCAM^{+}$ and Beta $Tubulin^{+}$ < 15% and $A2B5^{-}$ < 10% .

endodermal, ectodremal and mesodermal lineage. Growth factors such as bone morphogenic 4 (BMP4) and bFGF in CM-MSC can initiate differentiation of embryoid body (EB) into mesodermal lineage (osteogenic and chondrogenic) by increasing Wnt3 expression (Lee *et al*., 2014).

In this study, we successfully cultured NPCs in CM100 and CM50. This indicated that various growth factors in the CM-ratBMMSC were able to maintain NPCs growth and proliferation and also its differentiation. Based on immunoassay we found that CM-ratBMMSC contained specific growth factor, bFGF and NGF, in which the concentration of both growth factors was higher compared to other mediums used in this study (FM and NM) (Table 1).

Basic fibroblast growth factor (bFGF) and NGF can be secreted by MSC adipose derived or other tissue (Clauser *et al*., 2013). Basic fibroblast growth factor (bFGF) is one of the proteins that play a role in neurogenesis both in differentiation and proliferation (Woodbury and Ikezu, 2014). This growth factor usually added in cocktail growth factor and neurotrophic factor such as retinoid acid, FGF, insulin growth factor (IGF) to differentiate MSC to neuronal (Ahmedy *et al.,* 2015). Our result showed that CM50 can also be used for culturing of NPCs (Figure 2 and 3). It induced NPCs differentiations which were shown by positive expression of beta-tubulin marker after 4 days culture in both of gelatin and PDL coated culture plates. Gelatin and PDL used in this study were different extracellular matrix (ECM) that support NPCs culture. These results were also interesting secondary findings of this study that revealed there were differences between PDL and gelatin coating on neuronal cultures.

Extracellular matrix (ECM) is one of supporting factors for NPCs culture. Choosing appropriate coating agent is very important for neuronal cultures. Poly-D-lysine (PDL) were strongly modulates the adhesion and morphogenesis of primary hippocampal neurons (Sun *et al*., 2012) We found that NPCs population and neuron (PSANCAM and beta-tubulin markers) in PDL-coated was better than gelatin (Figure 2 and 3). This result was similar to previous study reported by Kim *et al.* (2011). They suggested that PDL has cell adhesion properties for cell growth and morphology, so that the number of neuronal cells, neurites per neuronal cell will be higher. They also reported that the neuronal cells on PDL bounded surfaces survived for longer time (Kim *et al*., 2011).

Our experiments that used three different mediums (FM, CM100 and NM) showed positive expressions of astrocytes marker (GFAP) and neuron (NeuN) (Figure 4 and Table 2). The number of cells with positive expressions of GFAP and NeuN from CM100 culture were higher than other groups which indicated there were specific substance in CM-rat BMMSC that play role in the neurogenesis in vitro. ELISA results confirmed this hypothesis in which there were higher concentration of bFGF and NGF in that CM compared to FM and NM.

Fibroblast growth factor (FGF) has neurogenesis and neuroprotection effect for NPCs culture. Besides, nerve growth factor (NGF) and FGF in CM-ratBMMSC may activate phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) pathway that is very important for NPCs activity. This pathway controls neurogenesis such as proliferation, migration and differentiation of NPCs. (Koh and Lo, 2015) In addition, FGF will also binds FGF reseptor (FGFR). Signalling effects from that receptor binding not only important for neurogenesis but also for synaptic formation, neuroglia interactions, inflammation, and amyloidosis and also gives neuroprotective effects in hippocampal region by suppressing autophagy via mTOR pathway and inhibiting apoptosis by preventing p53 (Woodbury and Ikezu 2014); (Sun *et al*., 2018)

Conditioned medium from MSC may not only contain growth factors but also cytokine, chemokine and extracellular vesicles that potential for neurodegenerative therapy (Cantinieaux *et al*., 2013); (Li *et al*., 2018)[2] (Henry *et al*., 2018) Extracellular vesicles (EVs) such as exosomes and microvesicles which contained a variety of cargo including proteins, lipids, DNA and various RNA can modulate signaling pathway to treat ischemic stroke (Li *et al*., 2018) Growth factor, neurotrophic factor and EVs induced neuro restorative, increasing neurogenesis, synaptogenesis, anti-inflamatory and angiogenesis, suppressing apoptosis and inflamation (Henry *et al*., 2018; Manuel *et al*., 2017). The findings of this research, using CM-ratBMMSC in NPCs culture, may open up the opportunities of cell-free therapy for neurodegenerative disease.

CONCLUSION

Growth and neurotrophic factor such as bFGF and

NGF from CM-rat BMMSC increased the differentiation ability of NPCs into astrocytes (GFAP) and neurons (NeuN).

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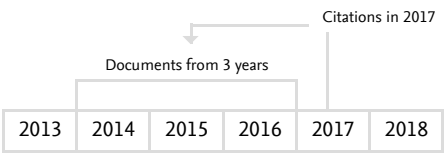

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