Immunocytochemistry Studies Using Microtubule-Associated Protein-2 (MAP-2) Markers on Neural Differentiation of Mesenchymal Stem Cells from Rat Bone Marrow

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Abstract. Mesenchymal stem cells (MSCs) can differentiate into neurons, which can be used in cell therapy. Endogenous genes and neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 regulate neural stem cell proliferation and differentiation (NT-3). The differentiation of MSCs into neurons is characterized by the development of neurons that interact with each other to form rings or net-like structures. The markers of neurons in the immature stage are Nestin and Vimentin. While the markers for neurons at the mature stage are neuron-specific enolase (NSE), neuronal-specific nuclear protein (NeuN), and microtubule-associated protein-2 (MAP-2). This research aims to investigate the expression of MAP-2 on the differentiation of MSCs into mature neurons. MSCs were isolated from rat bone marrow of femur and tibia using flushing methods, then cultured in Minimum Essential Medium Eagle (MEM), 10% Fetal Bovine Serum (FBS), and 1% antibiotic-antimycotic. Neuron differentiation induction medium (MEM, 2% FBS, 1% insulin-like growth factor (N2), and NT-3 20, 25, and 30 ng/mL) was used to stimulate MSCs for 14 days (control induction medium without NT-3). The immunocytochemistry of MAP-2 was performed on day 14. All experiments were done triplicated. The data obtained is the average percentage of the number of MAP-2 positive cells at each concentration. SPSS statistical analysis with a one-way ANOVA test. The results showed a significant difference between the percentage of MAP-2 positive cells at NT-3 concentrations 20 ($p < 0.05$), 25 ($p = 0{,}093$), 30 ng/mL ($p = 0{,}081$), and the negative control. At concentrations of NT-3 20, 25, and 30 ng/mL the

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results showed a significant difference in the percentage of MAP-2 positive cells 13.82 ± 1.61%, 11.50 ± 1.38%, 11.34 ± 1.29% versus the control 8,24 ± 0.70%, respectively. The study confirms that MSCs can differentiate into mature neurons and can be confirmed by the MAP-2 marker and 20 ng/mL NT-3 could be used as a single inducer of MSCs differentiation into the mature neurons, indicated by the presence of MAP-2 marker.

Keywords: Differentiation · Immunocytochemistry · Microtubule-Associated Protein-2 · Mesenchymal Stem Cells · Neuron

1 Introduction

The mesenchymal stem cells (MSCs) can differentiate into neurons that can be used for cell therapy. Transplantation using MSCs is being used to restore neuron function after neurodegenerative diseases like Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), Multiple Sclerosis (MS), and Amyotrophic Lateral Sclerosis (ALS) [1], Brain atrophy and abnormal protein deposition are common features of neurodegenerative diseases, which also cause a progressive decline in neuronal function. Several studies found that MSCs transplantation improved various models of neurodegenerative disease insults in animal models. MSCs transplantation frequently increased the regeneration and survival rates of neurons, reduced pathology, and reversed cognitive function decline [2, 3].

MSCs can self-renew, remain undifferentiated, and differentiate into various types of cells [4, 6]. MSCs are also multipotent and able to multiply and differentiate into a variety of various types of body cells, including osteoblasts, chondrocytes, adipocytes, and neurons. The ability of MSCs to differentiate into a variety of cell types gives them the potential to be used as treatment cells, particularly for neurological illnesses. Adult tissues such as bone marrow, adipose tissue, peripheral blood, cord blood, tendons, and ligaments can all be used to obtain MSCs. MSCs from the bone marrow are more invasive and can be acquired in modest quantities [4]. The ability of MSCs from bone marrow to develop into neurons has also been observed [5, 6].

The proliferation and differentiation of MSCs to neural stem cells are regulated by endogenous genes, neurotrophic factors, growth factors, and chemicals. Adding growth factors to the culture medium, such as bone morphogenetic protein-4 (BMP-4), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) cause MSCs to differentiate into neurons [7, 8]. Other chemical substances that have been reported to induce neural differentiation include β-mercaptoethanol (BME), forskolin (FSK), and retinoic acid (RA) [9, 10]. Several neurotrophic factors that are important in inducing the proliferation and differentiation of neural stem cells are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) [11, 12].

Immunocytochemistry analysis can be used to characterize the outcomes of MSC differentiation into neurons in vitro. Neuronal development can be observed with specific markers. The immature neuron can be characterized by polysialylated-neural cell adhesion molecule (PSAN-CAM), doublecortin, and beta-III-tubulin (Tuj1). Nestin and Vimentin as neuron progenitor markers. The mature neuron can be characterized by neuron-specific enolase (NSE), O4 (oligodendrocyte marker), neuronal-specific nuclear protein (NeuN), or microtubule-associated protein-2 (MAP-2). Glial fibrillary acidic protein (GFAP) and A2B5 (glial cell progenitor markers) are glial cell markers (Rushing and Ihrie 2016). The induction of MSCs into neurons is also characterized by the formation of neurons that grow slowly and connect to form rings or structures such as nets, as confirmed by the MAP-2 marker. MAP-2 is a cytoskeletal protein that is required for neuronal proliferation, development, differentiation, and maintenance [13, 14]. MAP-2 is important for the stability of dendritic structural integrity and neurite growth because it binds with microtubules to stabilize the neuronal cytoskeleton [15].

Previous research has shown that a combination of several growth factors and neurotrophic factors plays a role in neuron differentiation. Using a mixture of growth factors, including bFGF, EGF, IGF-1, and NT-3, Guan et al. (2014) showed that MSCs from rat bone marrow could differentiate into neurons [8]. According to Zhu et al. (2012), NT-3 promotes the differentiation of rat neural stem cells into neurons and oligodendrocytes during the 16-day gestation stage [9]. Rinendyaputri et al. (2018) reported that growth factors including bFGF and NGF were present in conditioned medium-mesenchymal stem cells (CM-MSCs), which can prevent neuronal cells from degenerating and promote the differentiation of neural stem/progenitor cells (NPCs) into astrocytes and neurons [16]. CM from neural progenitor cells (NPC) is also reported can stimulate differentiation to astrocytes and neurons and can maintain cell growth [17, 18].

Noviantari *et al.* (2020) showed that the addition of bFGF, EGF, and BDNF to the medium induced the differentiation of rat bone marrow MSCs into neurons and glia and characterized neural markers like A2B5, beta-III-tubulin, PSA-NCAM, and MAP-2) [19]. Noviantari *et al.* (2020) also reported that NT-3 role as a single neurotrophic factor in the expression of nestin in the immature neural differentiation of MSCs [20]. A combination of growth factors and neurotrophic factors consistently demonstrated efficacy in neural differentiation protocols. Furthermore, Schartz et al. (2016) reported that there is a correlation between MAP-2 loss and reactive microglia during refractory epilepsy. These MAP-2 studies are the starting point to determine potential roles for microglia in the dendritic pathology linked to epilepsy [15]. However, the expression of MAP-2 as a marker of mature neurons also has not been widely studied in neural induction medium with a single neurotrophic factor in vitro. So, this research aims to investigate the expression of MAP-2 on the differentiation of MSCs into mature neurons in a neural induction medium with a single neurotrophic factor.

2 Material and Methods

2.1 Rat MSCs Isolation, Culture, and Identification

Rat MSCs were isolated from the tibias and femurs of adult Wistar rats (*Rattus norvegicus L*) as previously described by Rinendyaputri *et al.* (2015) and Noviantari *et al.* (2020) [21,

22]. All procedures involving the use of animals were approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia. Cells were maintained in an Eagle's® minimum essential medium (MEM) supplemented with penicillin-streptomycin (10,000 U/mL) and 10% fetal bovine serum (FBS). Then the aspirate was incubated in a 5% CO_2 incubator at 37 °C. After 24 h, non-adherent cells were removed and adherent cells were cultivated in a fresh culture medium. Then, adherent cells were grown to a confluency of approximately 70–80%. Every 2–3 days, the medium was replaced.

Passage 4 (P4) was used for the differentiation assay. The differentiation assay was using a commercially available kit [23]: StemPro Adipocyte Differentiation Kit (Gibco A1007001), StemPro Chondrogenesis Differentiation Kit (Gibco A1007101), and StemPro Osteogenesis Differentiation Kit (Gibco A1007201). Every three days, the medium was changed. On day 14, osteoblasts, chondrocytes, and adipocytes were identified using Alizarin red staining (Sigma A5533), Alcian blue (Sigma A3157), and Oil red O staining (Sigma O0625), respectively.

The antibodies used to identify rat MSCs were anti-CD90-allophycocyanin (APC) (Biolegend 202526), anti-CD29-fluorescein isothiocyanate (FITC) (Biolegend 102206), and anti-CD45-phycoerythrin (PE) (Biolegend 202207). Immunophenotype analysis of rat MSCs was conducted using a BD AccuriTM C6 Plus flow cytometer according to the instruction kit.

2.2 Neuronal Induction and Differentiation

Rat MSCs were harvested by trypsinization using trypsin ethylenediaminetetraacetic acid (EDTA) and plated in a 24-well plate with a cell concentration of 2×10^4/mL. The neural induction and differentiation techniques of MSCs were altered from those of Noviantari et al. (2020) [20]. The cells were cultured in an induction medium (MEM) with 1% N-2 supplements, 2% FBS, and NT-3 with concentrations of 20, 25, or 30 ng/mL in each well. The induction medium without NT-3 was used as a control well. Then, the cells were incubated in a 5% CO_2 incubator at 37ºC. The induced rat MSCs were observed under a microscope and the medium was replaced every 2–3 days.

2.3 Immunocytochemistry Staining to Identify the Neural-Induced Rat MSCs

The immunocytochemistry staining to identify the neural-induced rat MSCs was conducted on day 14. The staining protocol was adopted by Noviantari, et al. (2020) [19]. The culture medium was removed from the well-plate, then rinsed with PBS twice. The cells were fixed with 4% paraformaldehyde (PFA) for 15 min and rinsed with PBS 3 times each for 5 min. Endogenous cell peroxidase was inhibited using blocking procedures so that it wouldn't interfere with the staining procedure. During this technique, background staining was blocked with 3% (v/v) H_2O_2 in methanol for 15 min and a background snipper (Starr Trek Universal HRP Detection Kit, Biocare).

After discarding the blocking reagents, the cells were washed three times in PBS for five minutes each time before being incubated with MAP-2 primary antibody (Santa Cruz) at 4ºC overnight. The cells were washed in PBS three times for five minutes. Then the cells were incubated for a further 15 min with secondary horseradish peroxidase (HRP)-conjugated antibody before being washed in PBS for 5 min. Following a 15-min incubation with Trek-Avidin-HRP, they were washed in PBS for 5 min. The cells were treated for 1–2 min with chromogen substrate diaminobenzidine (DAB) dissolved in substrate buffer before being washed with Milli-Q water for 10 min. The cells were counterstained for 1–2 min with Hematoxylin Mayer, then the cells were washed in Milli-Q water for 5 min. The cells were examined with a Nikon inverted microscope (ECLIPSE TE2000-U).

Every staining process had both positive and negative controls. The primary isolated neuron culture from the rat brain served as the positive control for MAP-2 immunocytochemistry. The positive control is used as quality control of neuron immunocytochemistry staining. Brown color in the cytoplasm and nucleus region was used to identify MAP-2-positive cells. The immunocytochemistry procedure without MAP-2 primary antibody incubation served as the negative control. MAP-2-negative cells were those with a bluish nucleus or a hematoxylin-stained nucleus.

2.4 Cell Counting

The presence of MAP-2-positive cells was counted from triplicates well (NT-3 20, 25, and 30 ng/mL) (Fig. 1) in five randomly assigned high power magnification 400x fields. Images were captured using an inverted microscope and the number of cells was calculated by Software Image Raster 3.0. The cells were examined by two independent researchers.

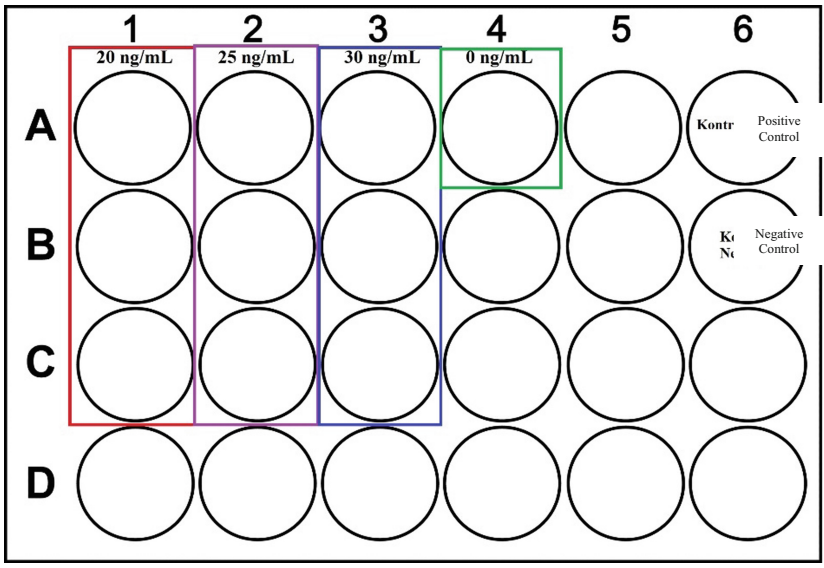

Fig. 1. Scheme of NT-3 concentration grading test on 24 well-plate

2.5 Statistical Analysis

Data are presented as the means of the percentages of MAP-2-positive and negative cells ± the standard error of the mean (SEM) of separate experiments. Statistical comparisons were done using a one-way analysis of variance (ANOVA) with Statistical Product and Service Solution (SPSS) 16. Least Significant Difference (LSD) post-Hoc analysis was used to identify the pair groups that carried significant associations. Significant differences are indicated as $p < 0.05$.

3 Results

3.1 Rat MSCs Isolation, Culture, and Identification

Rat MSCs were successfully isolated from the bone marrow of rat femurs and tibias. The cells in passage 4 have morphological-like mesenchymal stem cells with fibroblast-like cells and have spindle-shaped. After one week, the adhering cells had 80% confluence and monolayer (Fig. 2A).

The differentiation assay of the rat MSCs into osteoblasts, chondrocytes, and adipocytes was tested. Rat MSCs were grown in an osteogenic induction medium,

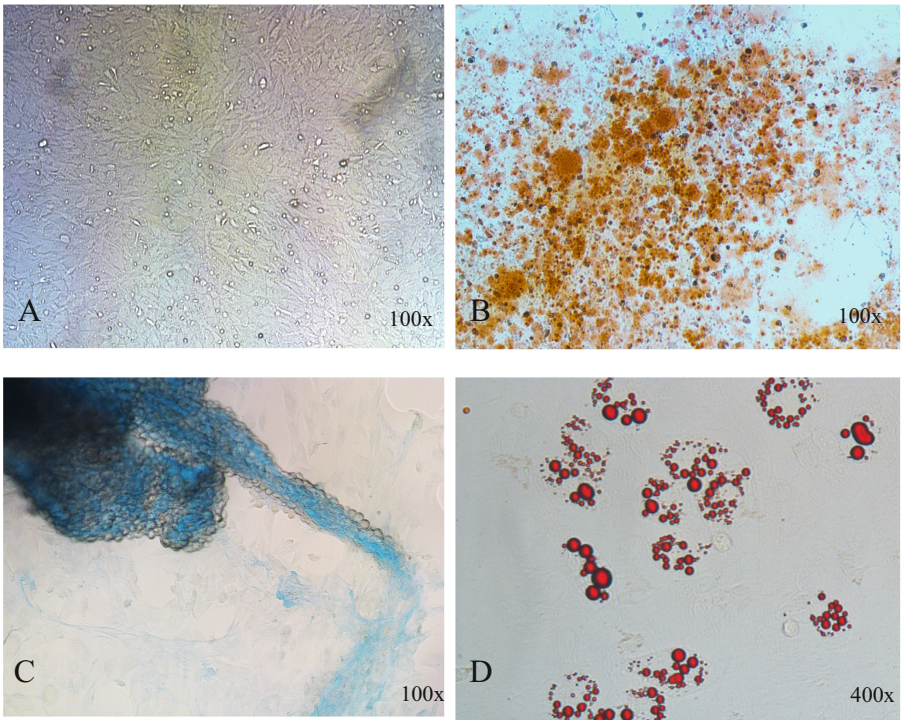

Fig. 2. Morphology of rat MSCs, A. Rat MSCs of the 4th passage before induction (100x), B. Rat MSCs differentiated into osteoblast (100x), C. Rat MSCs differentiated into chondrocyte (100x), D. Rat MSCs differentiated into adipocyte (400x)

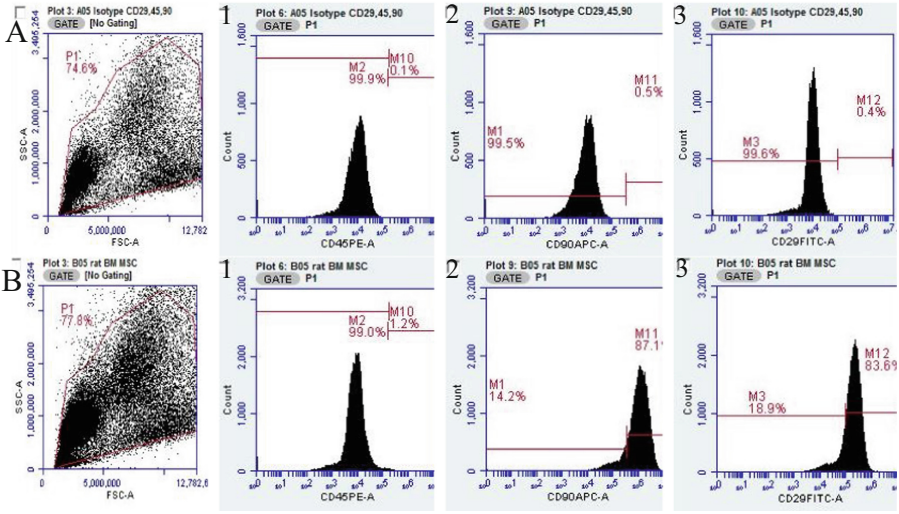

Fig. 3. Immunophenotype analysis using flow cytometry: A. isotype control and B. sample of rat MSCs passage 4 before neural induction

and alizarin red staining revealed that calcium was present in the extracellular matrix (Fig. 2B). In the chondrogenic induction medium, the rat MSCs showed the presence of chondrocyte extracellular matrix with a specific proteoglycan of cartilage by alcian blue staining (Fig. 2C). In the adipogenic induction medium, the rat MSCs showed the presence of lipid droplets in the cytoplasm by oil red O staining (Fig. 2D).

The immunophenotype of rat MSCs using flow cytometry analysis showed that the isotype antibodies were used to stain the control population (Fig. 3A). The characteristics MSCs phenotype were negative for CD45 (1.2%) (Fig. 3B1), positive for CD90 (87.1%) (Fig. 3B2), and positive for CD29 (83.6%) (Fig. 3B3).

3.2 Immunocytochemistry Staining to Identify the Induced Rat MSCs

After the rat MSCs cultured in a neuronal induction medium, immunocytochemistry with MAP-2 was done. In this study, the cells were stained positively, characterized by the formation of neurons that grow slowly and connect to form rings or structures such as nets as look at positive control for MAP-2 immunocytochemistry was the primary isolated neuron culture from the rat brain (Fig. 4A). Negative controls are used for the validation of immunocytochemistry and showed the blue nucleus (Fig. 4B). The cells became round or oval with extended and elongated cytoplasm (neurite-like) The cells had a shape like neuronal cells and were confirmed by the MAP-2 marker at NT-3 0 ng/mL (Fig. 4C), 20 ng/mL (Fig. 4D), 25 ng/mL (Fig. 4E), and 30 ng/mL (Fig. 4F).

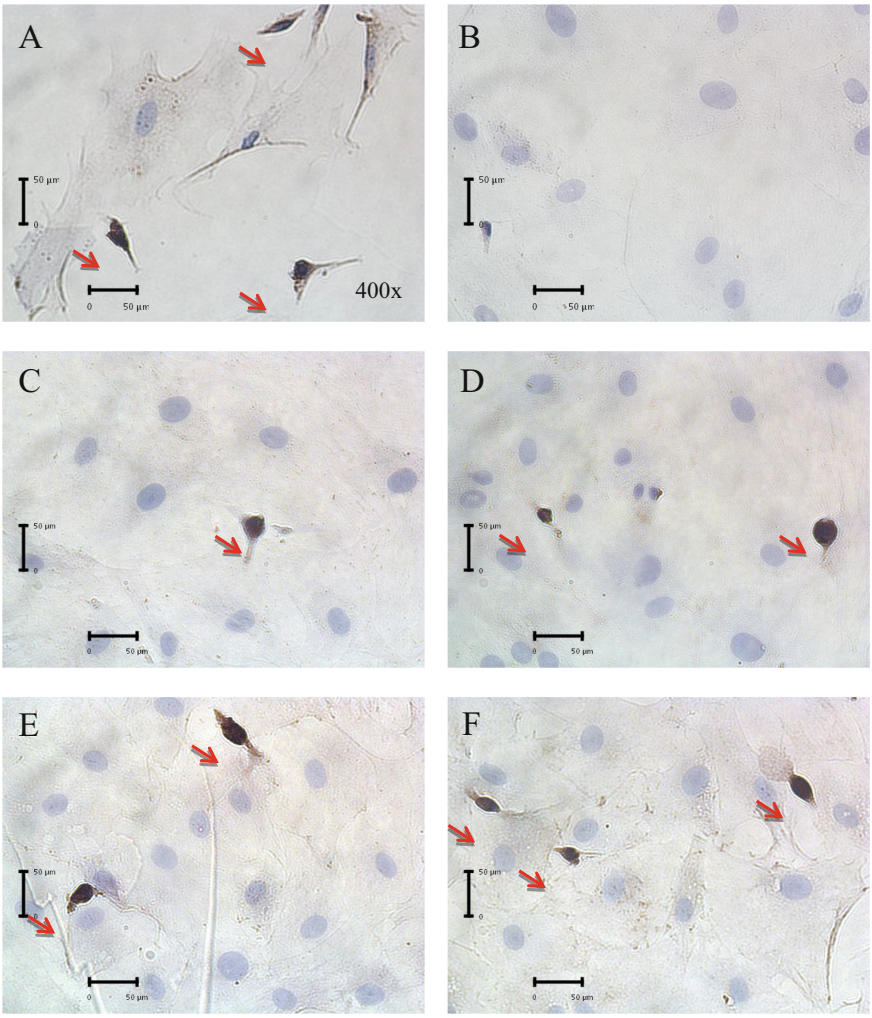

Fig. 4. Immunocytochemistry analysis of MAP-2 on day 14. A. positive control; B. negative control; C. NT-3 at 0 ng/mL; D. NT-3 at 20 ng/mL; E. NT-3 at 25 ng/mL; (F) NT-3 at 30 ng/mL. The arrow indicates MAP-2-positive cells. The positive control for MAP-2 immunocytochemistry was the primary isolated neuron culture from the rat brain (magnification 400x).

The results showed a significant difference in the percentage of MAP-2 positive cells and the control, as presented in Fig. 5. After Post-Hoc analysis using Least Significant Difference (LSD), MAP-2 expression on day 14 produced a statistically significant difference only observed between the control group and the treatment groups with additional NT-3 at 20 ng/mL ($p = 0{,}004$). The other group, 0 vs 25 ng/mL $p = 0{,}093$ and 0 vs 30 ng/mL $p = 0{,}081$.

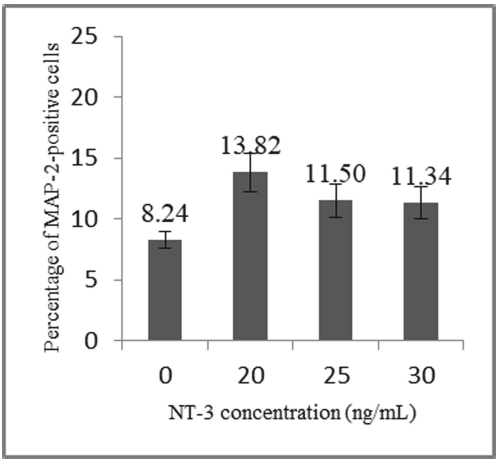

Fig. 5. Percentage of MAP-2 positive cells on day 14 with one-way ANOVA test.

4 Discussion

The isolation and culture of rat MSCs were successfully done in this study. After seeding and growing in culture plates, rat MSCs obtained from femurs and tibias of adult rats formed heterogeneous groups of cells. The suspended cells appeared to be small, round, and mixed with hematopoietic cells on initial plating. After 24 h, most of the cells subsequently adhered to the surface, expanded, and dispersed. After several days, adherent cells with spindle-shaped or large flattened morphology grew to confluence and displayed fibroblast-like morphology [24]. MSCs can differentiate into osteoblasts, chondrocytes, and adipocytes. Isolated MSCs expressed positive CD29, CD90, and negative CD45 MSC markers. Indeed, the MSCs used in this study fulfilled the International Society for Cellular Therapy's standard rat MSCs criteria (ISCT) [25, 26].

Inducing MSCs neural differentiation can be done by adding a combination of growth factors, neurotrophic factors, or several chemicals. Most of them used bFGF, EGF, NGF, and BDNF. Jahani et al. (2015) reported that inducing growth factors such as bFGF, BDNF, NGF, NT-3, and 3-isobutyl-1-methylxanthine (IBMX) in Dulbecco's modified Eagle's medium (DMEM)/F12 media tends to result in MSCs differentiation. Kil *et al.* (2016) reported that differentiation of the human WJ-derived MSCs into Neuronal Progenitor can be added by a medium containing EGF and bFGF for 14 days and differentiation into hair cells and neurons was induced using a neurobasal medium containing glial cell-derived neurotrophic factor (GDNF), BDNF, and NT-3 [27]. Chen *et al.* (2014) also found that combining NGF, BDNF, and bFGF significantly improved neural stem cells (NSC) proliferation and differentiation in the fetal brain of mice [12]. Guan et al. (2014) also noted that using a mixture of growth factors, including bFGF, EGF, IGF-1, and NT-3, rat MSCs can differentiate into neurons [8]. Noviantari *et al.* (2020) showed that the addition of bFGF, EGF, and BDNF to the medium induced the differentiation of rat bone marrow MSCs into neurons and glia [19].

Previous studies used a lot of combinations of growth factors, neurotrophic factors, or several chemicals, but rarely used a single factor for neuronal differentiation of MSCs. According to Zhu et al. (2012), NT-3 supports the differentiation of rat neural stem cells (NSCs) into oligodendrocytes and neurons during the 16-day gestation stage [9]. NT-3 is a protein member of the neurotrophin family that plays a role in neuronal survival, growth, and differentiation [28]. Neurotrophin also regulates synaptic structure, connection, neuroprotection, the growth of axons and dendrites, and glial cell development and function [29, 30]. The administration of neurotrophins is a potential approach to the therapy of neurodegenerative disorders or injuries such as Parkinson's disease (PD), Alzheimer's disease (AZ), spinal cord, brain trauma, and brain tumors [31]. Noviantari *et al.* (2020) reported that MSCs can be differentiated into neurons with a medium containing NT-3 as a single neurotrophic factor and expressed nestin in the immature neural differentiation of MSCs [20]. Thus, in this study, NT-3 was used to investigate the expression of MAP-2 in the mature neural differentiation of MSCs with a single neurotrophic factor.

Jiang et al. (2010) reported that several growth factors could cause MSCs to develop neuronal phenotypes. However, an immunocytochemistry study found that morphological ~~neuronal~~ characteristics of neurons and expressions of neuronal markers are insufficient indicators of neuronal differentiation [31]. The development of axons and dendrites that extended from the soma of the neurons, as well as connections between axons and dendrites, in induced rat MSCs into polarized neuron structures, showed gradual changes in the morphology of the cells that indicated neural differentiation. Neural differentiation markers are required for confirmation of neural differentiation [32, 33]. According to the stages of differentiation, there are many distinct indicators for the transformation of MSCs into neurons. MAP-2 was applied in this research as a marker for the mature neuron stage at the late phases of neural development [34–36]. MAP-2 is a marker for the ascent to higher cognitive function levels, and it suggests synaptic junction remodeling and neuroplasticity processes. A protein called MAP-2, which is abundant in dendrites, helps to stabilize the structure of neurons by encouraging the production of microtubules and forming cross-links with other cytoskeleton elements which is an essential step in neurogenesis. It suggests a part in determining and maintaining dendritic morphology during neuron development [37, 38].

The percentage of MAP-2-positive cells observed in this study was lower than that reported by Jiang et al. (2010) because of different growth and neurotrophic factor. In this study, only a single neurotrophic factor was added to the neural induction medium and showed mature neuronal markers with a range of 11–13% positive MAP-2 cells. Jiang, however, found that rat MSCs can differentiate into a neuron for 7 and 14 days in neural induction media made consisted of DMEM + 10%FBS, 10 ug/L bFGF, 10 ug/L EGF, 1 mmol dibutyryl cyclic AMP, and 0.5 mmol IBMX. Immunostaining analysis showed that the culture regimen exposed to cytokines expressed both immature neuronal markers (nestin, 40.17% ± 2.95%) and mature neuronal markers (MAP2, 36.30 ± 1.78%). The induced neuronal-like phenotypes are composed of mature neural cells expressing specific neuronal markers [31].

Mostafavi et al. (2014) found that after culturing in DMEM: F12 supplemented with 20 ng/ml human EGF, 20 ng/ml bFGF, and 2% B27, the mean percentage of MAP2

positive cells in rat MSC-derived neural-induced cells was 79.72% ± 2.2%. A normal mature neuron is identified by its cytological polarity, particular protein expression in certain regions, excitability, or the capacity to fire action potentials, as well as by the development of functioning synaptic structures that allow it to communicate with other cells [6].

MSCs can differentiate into mature neurons and can be confirmed by the MAP-2 marker in this current study. However, the study was limited by the absence of a particular marker for terminally differentiated glial and neuronal cells. Other research should include additional growth factors and characterize the neurons and glial cells generated by rat MSCs using markers such as NeuN, GFAP, and Olig-2. It is advised that tests on neural induction incorporate cellular functional observations such as electrophysiological parameters and cellular factors expression potentials in addition to morphological studies.

5 Conclusion

MSCs can differentiate into mature neurons and can be confirmed by the MAP-2 marker and 20ng/mL NT-3 could be used as a single inducer of MSCs differentiation into a mature neuron, indicated by the presence of MAP-2 marker.

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