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THE EXPRESSION OF NESTIN IN THE INDUCED DIFFERENTIATION INTO NEURONS OF RAT BONE MARROW MESENCHYMAL STEM CELLS BY NEUROTROPHIN-3 (NT-3)

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ABSTRACT

Objective: The aim of this study was to examine the role of NT-3 as a single neurotrophic factor in the expression of nestin in the neural differentiation of MSCs.

Methods: MSCs were isolated from rat bone marrow and induced with NT-3 at concentrations of 20, 25, and 30 ng/ml for 7 and 14 d (the control was no NT-3). Nestin underwent immunocytochemical analysis on days 7 and 14. Five high-power random fields were documented.

Results: A post-hoc analysis using LSD after one-way ANOVA test yielded a statistically significant difference in the percentage of nestin-positive cells in MSCs with NT-3 at concentrations of 20, 25, and 30 ng/ml for 7 d compared to the control group ($p<0.05$). The percentages of nestin-positive cells at concentrations of 20, 25, and 30 ng/ml, and in the control data on day 7 were 14.55±1.26%, 16.20±1.07%, 13.78±1.19%, and 9.81±0.79%, respectively. NT-3 at 25 ng/ml induced the highest MSCs neural differentiation on day 7 and remained constant until day 14.

Conclusion: NT-3 plays a role in the early stage of differentiating MSCs from rat bone marrow into neurons, with the optimal concentration being 25 ng/ml.

Keywords: Differentiation, Mesenchymal stem cells, Nestin, Neuron, Neurotrophin-3

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INTRODUCTION

Stroke is a cause of death amongst Indonesian citizens (>15 y old) that contributes to 15.4% of the total mortality rate (Riskesdas 2007) [1]. The prevalence of stroke increased to 12.1 per 1000 people in 2013, up from 8.3 per 1000 people in 2007 (Riskesdas 2013) [2]. Despite the advances in drugs that salvage the penumbra area of the brain, the stroke area succumbs from irreversible damage. Post-stroke neuronal deficit frequently occurs due to the limited capacity of the brain for neural regeneration [3]. Post-stroke neuronal deficits can potentially be recovered by stem cell transplantation. The capacity of stem cells to self-renew and to maintain stemness thus holds the potential for further differentiation into other lineages, including osteoblasts, chondrocytes, adipocytes, and neurons [4-5].

In vitro differentiation of mesenchymal stem cells (MSCs) into neurons has been reported by the combination of several growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), bone morphogenetic protein-4 (BMP4), fibroblast growth factor-8 (FGF-8). Other chemical substances, such as β-mercaptoethanol (BME), retinoic acid (RA), and forskolin (FSK), were also reported to induce differentiation into neurons [6, 7]. In addition, neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), were successfully documented as influencing *in vitro* differentiation of neural stem cells and MSCs into neurons [7, 8].

Neurotrophin-3 (NT-3), hippocampus-derived neurotrophic factor (HDNF), and NGF-2 each have a molecular weight of 13.6 kDa, which is identical to that of NGF and BDNF. NT-3 is a protein member of the neurotrophin family that plays a role in neuronal survival, growth, and differentiation [9]. Neurotrophin also regulates synaptic structure, connection, neuroprotection, the growth of axons and dendrites, and glial cell development [10, 11]. Zhu *et al.* (2012) reported that NT-3 contributes to the differentiation of neuronal stem cells of rats into neurons and oligodendrocytes at 16 d of gestation [6]. In addition, Guan *et al.* (2014) demonstrated that MSCs from rat bone marrow could differentiate into neurons using a combination of growth factors, i.e., EGF, bFGF, IGF-1, and NT-3 [8].

A combination of growth and neurotrophic factors consistently showed efficacy in neural differentiation protocols. However, the role of each factor, particularly NT-3 as a single neurotrophic factor, in MSCs neural differentiation is not yet completely understood. The aim of this study is to examine the role of NT-3 as a single neurotrophic factor in the expression of nestin in the neural differentiation of MSCs, in order to provide basic theoretical data for further research in managing neurodegeneration.

MATERIALS AND METHODS

Methods

This study was conducted from February to November 2016 at the Stem Cell Laboratory, Center for Research and Development of Biomedical and Basic Health Technology (CRDBBHT), National Institute of Health Research and Development (NIHRD), Ministry of Health in our country and at the Histology Laboratory in our university.

Isolation, culture, and characterization of MSCs from rat bone marrow aspirate

All procedures for handling rats were in compliance with the local animal welfare facility rules and were approved by the Ethics Committee FMUI no. 208/UN2. F1/ETIK/2016. Twelve Sprague Dawley rats (*Rattus norvegicus L*) from the Animal Laboratory, CRDBBHT NIHRD, aged 2–4 mo, and weighing 200–300 g were used in this study. Rats were anesthetized via intraperitoneal injection using a ketamine dose of 75–100 mg/kg in a mixture with a xylazine dose of 5–10 mg/kg in order to obtain the femur and tibia bones.

MSCs were isolated from the femur and tibia bones by following a modification of the method of Rinendyaputri *et al.* (2015) [12] by

adding povidone-iodine to decontaminate bones. First, the bones were immersed in 1% povidone-iodine solution and 70% ethanol antiseptic on a separate petri dish for two minutes. In addition, the attached fat and connective tissue were removed using a pair of tweezers and a scalpel. The bones were then immersed in Dulbecco's® phosphate-buffered saline (PBS) solution, prepared by diluting PBS 10x (Sigma®) 1:10 in Milli-Q water and filtering through a 0.2 mm sterile filter. Each bone end was sectioned using a pair of tweezers and scissors until the bone marrow was exposed. Subsequently, bone marrow aspirate was flushed using a 3 ml syringe that contained Eagle's® minimum essential medium-MEM (Sigma®) supplemented with penicillin-streptomycin (10,000 U/ml) (Gibco®) and 10% fetal bovine serum-FBS (Gibco®) into a petri dish while the bone was stabilized using a pair of tweezers. Then the aspirate incubated in a 5% CO_2 incubator (Heracell Vios 160i) at 37 °C. After 24 h, the medium was discarded. The subsequent medium was changed every 2–3 d.

Passages were initiated after the cell confluence reached approximately 70–80%. Passage 4 (P4) was used for the immunophenotype analysis, the differentiation assay, and the induction of neural differentiation. Immunophenotype analysis of MSCs markers with anti-CD29-fluorescein isothiocyanate (FITC), CD90-allophycocyanin (APC), and CD45-phycoerythrin (PE), all three provided from Biolegend®, was conducted using a flow cytometer (BD FACSCalibur®). A differentiation assay was performed according to the protocol supplied by the StemPro Adipocyte Differentiation Kit, StemPro Chondrogenesis Differentiation Kit, and StemPro Osteogenesis Differentiation Kit (all kits by Gibco®).

Induction of MSCs neural differentiation with NT-3

The induction methods of MSCs neural differentiation were modified from those of Guan *et al.* (2014) by changing Dulbecco's modified Eagle's medium with low glucose to MEM [9]. MSCs P3 was harvested by trypsinization using trypsin ethylenediaminetetraacetic acid-EDTA (Gibco®) and centrifuged. The pellet was resuspended in the induction medium (MEM-Sigma) containing 2% FBS (Gibco®) and 1% N-2 supplements (Gibco®). NT-3 (Sigma®) was added to the induction medium to obtain a final concentration of 20, 25, or 30 ng/ml in each well. The NT-3 concentration was optimized by a gradient dose concentration from 0–60 ng/ml (Appendix 1). The control well was cultured using a basic induction medium (MEM+2% FBS+1% N2). The MSCs were seeded on 24 well culture plates with a final cell density of 2×10^4/ml and incubated in a 5% CO_2 incubator at 37 °C. MSCs treated with three different concentrations of NT-3 were cultured in triplicates, respectively. The induction medium (with or without NT-3 supplementation) was replaced every 2–3 d. The induced MSCs were observed under a Nikon inverted microscope (ECLIPSE TE2000-U with Imaging Software NIS-Elements F Version 4.30.01). The immunocytochemical analysis of the nestin was conducted at day 7 and day 14.

The nestin immunocytochemistry of the induced MSCs

The immunocytochemical analysis of the MSCs induced neural differentiation was modified from Stephanie *et al.* (2013) by decreasing the time of incubation of the MSCs seeded on the well plate [13]. The culture medium was discarded from the well, leaving only the attached MSCs on the well-plate surface. These MSCs were then rinsed with PBS twice. The MSCs fixation was done by incubating these cells in 4% paraformaldehyde (PFA) for 15 min and then they were rinsed with PBS 3 times each for 5 min. We performed blocking steps in order to inhibit endogenous cell peroxidase so that this enzyme will not impair the staining process. This procedure was carried out using 3% (v/v) H_2O_2 in methanol (Merck®) for 15 min and (ii) blocking of nonspecific background staining with a background snipper (Starr Trek Universal HRP Detection Kit Biocare®) for 15 min. The blocking reagents were then discarded and MSCs were rinsed three times in PBS for 5 min each time, then incubated with nestin primary antibody (Santa Cruz®) at 4 °C overnight. The next day, the same cells were rinsed again three times in PBS for 5 min each time. Further incubation with a secondary horseradish peroxidase (HRP)-conjugated antibody (Trekkie Universal Link, Starr Trek Universal HRP Detection Kit Biocare®) lasted for 15 min and then these cells were rinsed in PBS for 5 min. After that, incubation with Trek-Avidin-HRP (Starr Trek Universal HRP Detection Kit Biocare®) took place for 15 min and then they were rinsed in PBS for 5 min. The chromogen substrate diaminobenzidine (DAB) dissolved in substrate buffer (Starr Trek Universal HRP Detection Kit Biocare®) was added into the cells for 1–2 min, and then these were rinsed with Milli-Q water for 10 min. After removal of Milli-Q water, these cells were counterstained with Hematoxylin Mayer (Biocare® 3570) for 1–2 min and finally rinsed in Milli-Q water for 5 min.

Positive and negative controls were included in every staining protocol. The positive control for nestin immunocytochemistry was the primary isolated neuron culture from the rat brain. Nestin-positive cells were indicated by a brown color in the cytoplasmic and nucleus area. The negative control was the immunocytochemistry protocol without the nestin primary antibody incubation. Nestin-negative cells appeared as cells with a bluish stained nucleus or a hematoxylin stained nucleus.

Nestin tissue expression data analysis

Nestin-positive cells were counted in triplicates from five randomly assigned high power magnification 400x fields. Images were documented under a Nikon inverted microscope (ECLIPSE TE2000-U) and Software Image Raster version 3.0 was used to calculate the number of cells tagged by the investigator. Nestin-positive cells (expressed in the nucleus and cytoplasm) and nestin-negative cells were tagged and counted by two investigators without prior knowledge of the sample label (double-blind). The percentages of nestin-positive and negative cells were measured from two sets of observations.

Data analysis

The percentages of nestin-positive and nestin-negative cells were compared among different experiment groups through a one-way analysis of ANOVA using Statistical Product and Service Solution (SPSS) 16. Post-Hoc analysis using LSD was performed to determine which pair groups carrying significant association. Significant differences are indicated as $p<0.05$. The results are provided as the mean±the standard error of the mean.

RESULTS

Isolation, culture, and characterization of rat bone marrow MSCs

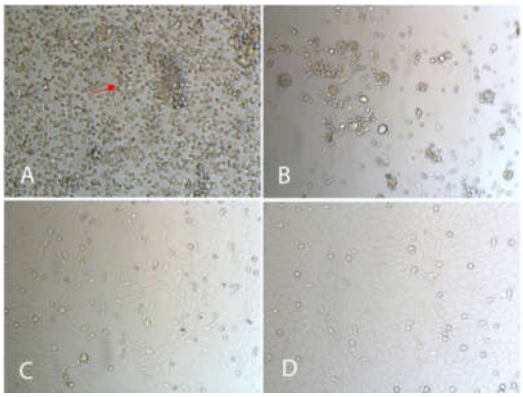

Fig. 1: Morphology of primary MSCs isolated from rat bone marrow, (A) Culture of MSCs isolated from rat bone marrow, day 0 (magnification 400x). The arrow indicates erythrocytes, (B) Culture of MSCs from rat bone marrow, day 1 (magnification 100x), (C) Culture of MSCs from rat bone marrow, day 5 (magnification 100x), (D) Culture of MSCs from rat bone marrow, day 8 (magnification 100x)

The isolation of MSCs from rat bone marrow initially showed a heterogeneous cell population, which was gradually populated by

MSCs-like morphology. On day 0, the cells in the primary bone marrow aspirate culture were globules of different sizes and were well distributed, and erythrocytes were still present in the well (the arrow in fig. 1A). On day 1, the cells started to attach to the culture well (fig. 1B). On day 5, the cells showed fibroblastic-like or spindle-shaped forms and the other cells showed globular cell forms of comparable size (fig. 1C). On day 8, the cells attached to the culture well as a single layer and reached confluency (fig. 1D). At this point, the standard MSCs morphology, i.e., their fibroblast-like shape, was observed. Subsequent passages were done until passage 4 in order to obtain an adequate number of cells for the experiments on inducing MSCs neural differentiation.

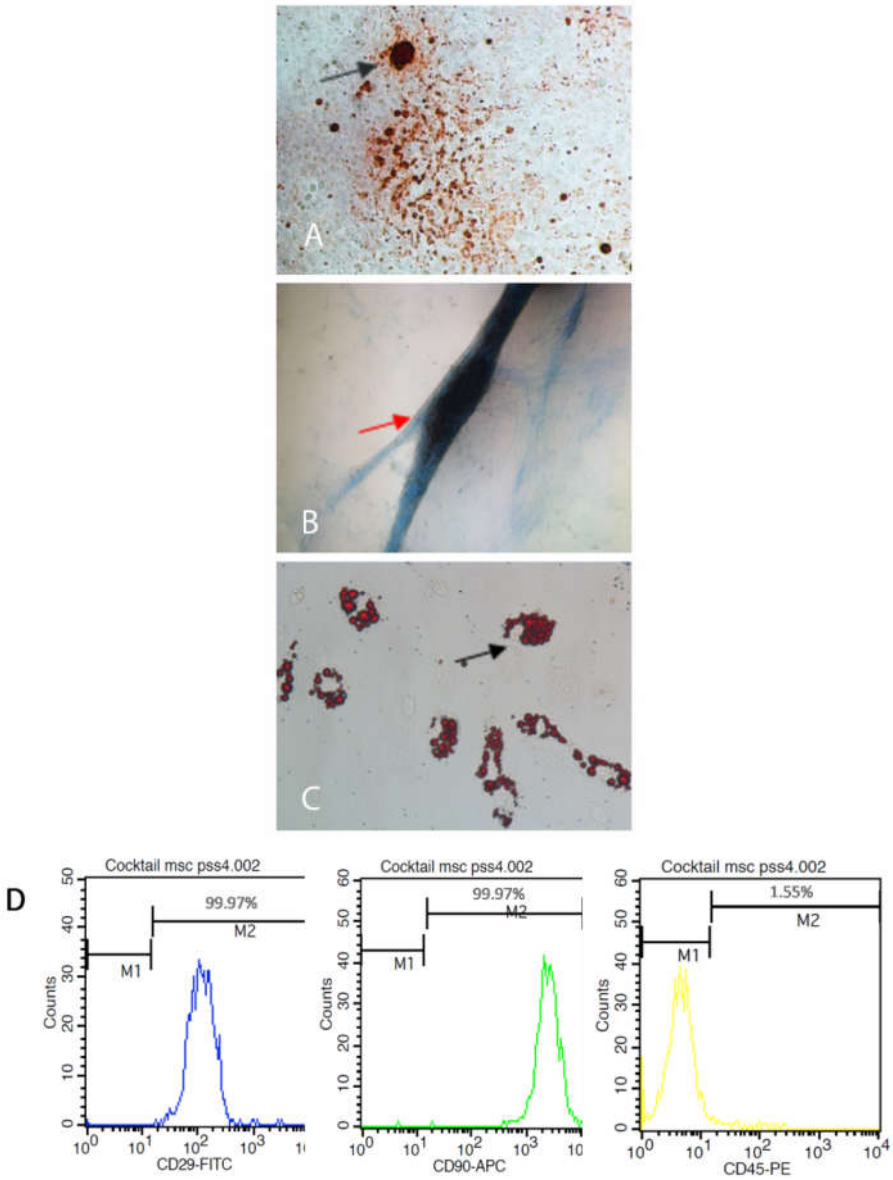

Fig. 2: (A) Differentiation potency of MSCs from bone marrow: osteoblast with the presence of calcium in the extracellular matrix (blue arrow) (magnification 100x); (B) chondrocyte with a specific proteoglycan of cartilage (red arrow) (magnification 40x); (C) adipocyte with lipid droplets inside the adipocyte cytoplasm (black arrow) (magnification 400x); (D) and phenotype of MSCs from bone marrow with flow cytometry analysis

The differentiation potency of the MSCs was tested using a commercially available kit [14]. The MSCs isolated from the rat bone marrow were able to differentiate into osteoblasts, chondrocytes, and adipocytes. A histology analysis revealed the presence of calcium in the extracellular matrix by alizarin red staining, the presence of chondrocyte extracellular matrix by alcian blue staining and the presence of lipid droplets inside the adipocyte cytoplasm by oil red O staining (fig. 2A, B, and C). The characteristics of the immunophenotype of MSCs-P4 from bone marrow using flow cytometry analysis showed 99.97% CD29+CD90+(MSCs marker) and 1.55% CD45+/low (hematopoietic markers) (fig. 2D).

NT-3 at 25 ng/ml induced the highest MSCs neural differentiation on day 7 and was stationary on day 14

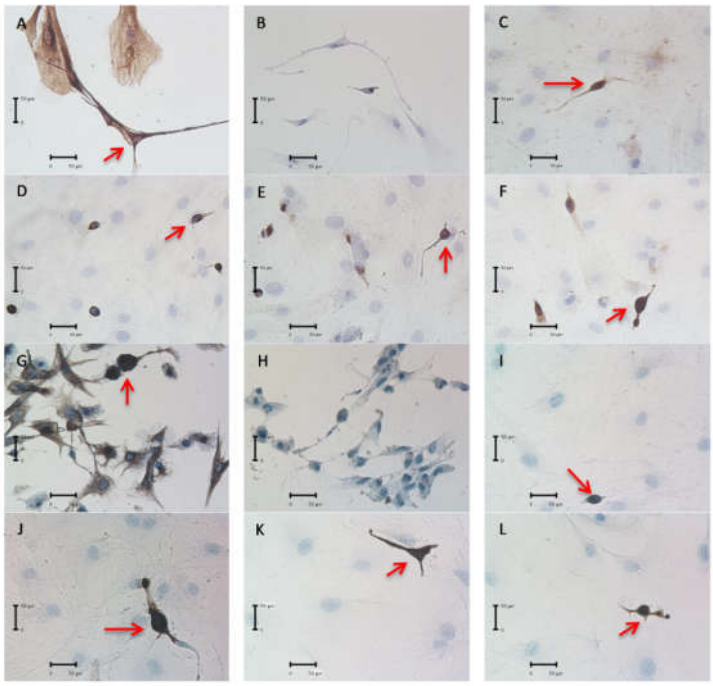

Fig. 3: Immunocytochemical analysis of nestin from day 7 (A–F) and day 14 (G–L); (A, G) positive control; (B, H) negative control; (C, I) NT-3 at 0 ng/ml; (D, J) NT-3 at 20 ng/ml; (E, K) NT-3 at 25 ng/ml; (F, L) NT-3 at 30 ng/ml. The arrow indicates nestin-positive cells (magnification 400x)

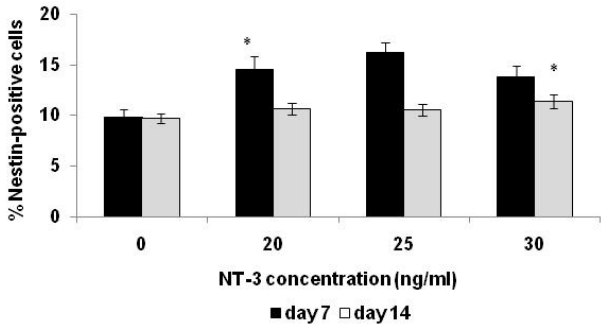

Fig. 4: Mean percentages of nestin-positive cells on day 7 (black histogram) and on day 14 (grey histogram). The error bar indicates the standard error of the mean (SEM), *represents a statistically significant difference, p<0.05 (n=15, compared with NT-3 group at concentration 0 ng/ml, n=10)

The histological images of induced MSCs neural differentiation were observed on day 7 and day 14 for NT-3 at 20 ng/ml (fig. 3D and 4J), at 25 ng/ml (fig. 3E and 3K), and at 30 ng/ml (fig. 3F, 3L), for no NT-3 (fig. 3C and 3I), for the positive control (fig. 3A and 3G), and for the negative control (fig. 3B and 3H).

Compared to the control group (NT-3 at 0 ng/ml) on day 7, percentages of nestin-positive cells treated with NT-3 at 20, 25, 30 ng/ml increased by 1.48 fold, 1.65 fold, and 1.4 fold, respectively (fig. 4). The percentages of nestin-positive cells at concentrations of 20, 25, and 30 ng/ml, and in the control data on day 7 were 14.55±1.26%, 16.20±1.07%, 13.78±1.19%, and 9.81±0.79%. Hence, the highest increment in the percentage of nestin-positive cells on day 7 was achieved with the addition of NT-3 at 25 ng/ml. After Post-Hoc analysis using Least Significant Difference (LSD), nestin expression on day 7 produced a statistically significant difference between the control group and the groups with additional NT-3 at 20, 25, and 30 ng/ml ($p<0.05$).

Meanwhile, on day 14, the mean percentages of nestin-positive cells with additional NT-3 at 20, 25, and 30 ng/ml when compared to the control group (NT-3 0 ng/ml) showed increments ranging only from 1.09-fold to 1.17-fold. The percentages of nestin-positive cells at concentrations of 20, 25, and 30 ng/ml, and in the control data on day 14 were 10.65±0.56%, 10.59±0.55%, 11.42±0.72%, and 9.71±0.47%. Using the same Post-Hoc analysis LSD, statistical significance ($p<0.05$) was only observed between the control and treatment group with NT-3 addition at 30 ng/ml. From these results, as illustrated in fig. 4, the mean percentages of nestin-positive cells on day 14 yielded lower proportions of nestin-positive cells, respectively for each group compared to those on day 7.

DISCUSSION

In this study, isolated MSCs from rat bone marrow aspirates were successfully maintained. The cell population expressed the MSCs markers $CD29^{+/high}$, $CD90^{+/high}$, and $CD45^{low}$, and differentiation tests

revealed the ability of MSCs to differentiate into osteoblasts, chondrocytes, and adipocytes. Indeed, the MSCs used in this study showed conformity to standard rat bone marrow MSCs [15].

Inducing MSCs neural differentiation with additional NT-3 in combination with NGF and BDNF has been reported by Jahani *et al.* (2015) [16] and Kil *et al.* (2016) [17], also in combination with other growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) IGF-1 [6]. This study investigated the role of NT-3 as a single neurotrophic factor in inducing MSCs neural differentiation. It is important to investigate NT-3 as a single neurotrophic factor in order to determine the significance of the factors that induce MSCs' neural differentiation. Induced MSCs neural differentiation was indicated by gradual changes in the induced MSCs morphology into polarized neuron structures, such as the formation of axons and dendrites that projected from the neuron soma or as connections between axons and dendrites (synapse formation).

Confirmation of neural differentiation requires neural differentiation markers. Nestin is well known as an intermediate filament protein in mammalian brain neural stem cells and neural progenitor cells during the early development of the central nervous system and proliferating neuroepithelial cells [18–20]. Wiese *et al.* (2004) [21] and Lindsay *et al.* (2016) [22] suggested nestin as a marker of progenitor cell multipotency. This study used nestin as a marker for the early stages of neural differentiation.

Nestin expression on day 7 produced a statistically significant difference between the control group and treated groups with NT-3 addition at 20, 25, and 30 ng/ml. This result indicates the importance of additional NT-3 in early-stage induced MSCs neural differentiation (7 d). The highest mean percentage of nestin-positive cells was obtained with additional NT-3 at 25 ng/ml.

The percentage of nestin-positive cells on day 7 was in line with the results from the study by Jeon *et al.* (2007) [23]. They showed induced MSCs neural differentiation with additional NT-3 at 30 ng/ml and bFGF at 10 ng/ml after 7 d; they obtained 14.2±2.0% as the percentage of nestin-positive cells.

The percentage of nestin-positive cells observed in this study was lower than that reported by Zhu *et al.* (2012) [6] because of different sources of MSCs. Zhu *et al.* induced primary isolated neural stem cells from the rat brain, while our study used MSCs from rat bone marrow. Neural stem cells have a more direct tendency to differentiate into neuro-ectodermal cell lines. Thus, inducing MSCs, which are of mesodermal characteristics, into neural cells, requires some alteration from their inherent differentiation pattern.

The results of our study support the role of NT-3 as a single neurotrophic factor that regulates early-stage neural differentiation. The signaling pathway regulated by NT-3 during neural differentiation has been elucidated in several studies [22–26]. NT-3 binds to the receptor neurotrophin tyrosine kinase (TrkC), which subsequently induces transphosphorylation of intracellular tyrosine residue and binds to Shc (Src [Rous sarcoma oncogene cellular homolog-an intracellular protein-tyrosine kinase lacking direct contact with extracellular signaling molecules] homology 2 domain-containing). NT-3 binds to other domains of the receptor, which activates growth factor receptor-bound protein 2 (Grb2), which binds to Sos (son of sevenless guanine nucleotide exchange factor) and subsequently forms a complex with Shc as Grb2-Sos-Shc. This complex activates Ras (oncogene protein P21), which then activates rapidly accelerated fibrosarcoma 1 (Raf-1) and the phosphorylates MAP/ERK kinase (MEK1) pathway. This pathway plays a significant role in cell survival, proliferation, and differentiation. Differentiation of embryonic stem cells and neural stem cells into neurons is regulated by this pathway [24,25].

In order to determine the optimum NT-3 concentration, this study initially used gradient dose concentration and compared each dose concentration to the percentage of viable MSCs in the well (data not shown). Further study is required to confirm which signaling pathway of NT-3 induced MSCs early-stage neural differentiation.

There was little effect of NT-3 on inducing MSCs neural differentiation after day 7. Extension of additional NT-3 in inducing MSCs neural differentiation from day 7 until day 14 showed a lower percentage of nestin-positive cells on day 14 than day 7. This result was in line with a study by Zhu *et al.* that showed a lower percentage of nestin-positive cells after additional NT-3 up to 14 d. The decrease in the percentage of nestin-positive cells is explained by the concurrent increase in the percentage of terminally differentiated neuron/glial cells.

The limitation of this study was the lack of a specific marker for terminally differentiated neuron/glial cells. Thus, we recommend further evaluation of the NT-3 role in terminal differentiation of specific neuron/glial cells (astrocytes and oligodendrocytes) by additional markers, e. g., MAP-2, GFAP, and Olig-2.

CONCLUSION

NT-3 at 25 ng/ml induced the highest MSCs neural differentiation on day 7. Differentiation was stationary by day 14. Thus, NT-3 plays a role in the early stage of differentiation into neurons from MSCs in rat bone marrow. The NT-3 concentration at 25 ng/ml in this experiment setting yields optimal results for neural differentiation into nestin-positive cells. This information provides insight for tackling neuroregeneration in neurodegenerative diseases and should be confirmed in further investigations on a larger scale.

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Nil

AUTHORS CONTRIBUTIONS

All the author have contributed equally.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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