ORIGINAL ARTICLE

DEVELOPMENT OF NEURITE OUTGROWTH FOLLOWING DIFFERENTIATION OF MOTOR NEURON-LIKE CELL LINE, NSC-34 BY DIFFERENT CONCENTRATIONS OF RETINOIC ACID.

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Abstract
Background: Glutamate excitotoxicity is one of the critical causes of motor neuron degeneration, a common hallmark in movement-related disorders. For the past decades, experimental model systems have been focusing on using animal models to elucidate the pathophysiology of neurodegeneration. However, the usage of animals is costly, time-consuming, and has ethical implications. Cell culture models such as the NSC-34 cell line, a mouse motor neuron-like hybrid cell, can serve as an alternative study system to overcome these limitations. NSC-34 cell line can be differentiated to express motor neuron properties using simple chemical treatments. The study reports on optimizing the differentiation protocol for NSC-34 cells using retinoic acid treatment.
Methods: NSC-34 cells were seeded onto a 12-well plate and grown in retinoic acid-containing media (1µM or 10µM). Cells were incubated for seven days in a humidified atmosphere with 5% CO_2 at 37°C. Cells were analyzed using an immunocytochemical technique for Beta-III tubulin (neuronal biomarker) and DAPI (nuclei marker). Cells were imaged were using phase-contrast microscopy and fluorescence microscopy.
Results: Neurite outgrowth was evident in both retinoic acid concentration groups. However, after seven days in culture, the projection of neurites in the 10µM retinoic acid group was more extensive than the 1µM group.
Conclusion: The present study supports the use of retinoic acid treatment at 10µM concentration as an efficient differentiation of NSC-34 cells into motor neurons. This method sees its application in experimentation for studying axonal transport and electrical impulse conductivity.

Keywords: NSC-34, retinoic acid, differentiation, in-vitro model, neurite development.

Introduction

Glutamate excitotoxicity is one of the critical causes of motor neuron degeneration in spinal cord injury. The secondary damage progression following the initial impact on the spinal cord involves the abnormal elevation of glutamate release, causing glutamate-mediated excitotoxicity.[1,2,3] Importantly, the excessive level of extracellular glutamate creates a cytotoxic environment at the site of injury, triggering a cascade of cell death processes.[1,4,5] The cascade of events associated with glutamate excitotoxicity include failure in calcium homeostasis, mitochondrial dysfunction, the release of reactive oxygen species and reactive nitrogen species, and high generation of oxidative stress, which eventually trigger cell death through apoptosis.[3,6,7]

For the past decades, in-vitro model systems have been developed and used to elucidate underlying mechanisms of neuropathies. One of the commonly used models for nerve injury and regeneration study is the primary neuron culture.[3,8,9] Primary neurons can be extracted from the animals such as rats and mice. Primary neurons can replicate the morphological and physiological properties of the native cells, making them more reliable as an experimental model system.[9,10,11] However, primary neuron culture has significant limitations like low cell yield during isolation, low cell purity, and inability to expand.[9,12]

NSC-34 cell line, a hybrid cell line generated by the fusion of mouse spinal cord neurons and mouse neuroblastoma, can be used to study the pathophysiology of motor neurons. With the capabilities to proliferate and produce clonally uniform cells and the easy availability of the cell line, the use of NSC-34 could overcome many challenges associated with isolating primary neurons from animals.[13,14,15] NSC-34 consists of two cells populations; (i) small undifferentiated cells derived from murine spinal motor neurons and; (ii) large multinucleated neuroblastoma cells with the capacity to proliferate and go through cell division.[16] Upon differentiation with the aids of differentiation agent, NSC-34 may exhibit the unique properties of mature motor neurons in the spinal cord, including the formation of long neurites and expression of neuronal markers such as Beta-III tubulin and choline acetyltransferase (ChAT).[15] Differentiation of NSC-34 requires modification of the culture media with the low serum concentration and enriched with differentiation agents such as retinoic acid (RA). Several studies have supported the use of RA to differentiate NSC-34 into motor neuron-like phenotypes.[12,15,17,18] However, the RA concentration used for NSC-34 differentiation differs from one study to another. Classically, 1µM of RA is commonly used to differentiate NSC-34 cells for seven days in-vitro.[15,18,19] However, recent studies have been increasing the concentration of retinoic acid to 10µM, differentiating the cells for a similar number of days. Therefore, this study compares the neurite outgrowth of differentiated NSC-34 cells using two different concentrations of RA, 1µM and 10µM.

Methodology

Differentiation of NSC-34

NSC-34 cell line was purchased from Cedarlane Laboratories. The cell line was cultured in a T75 flask containing Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% of penicillin-streptomycin (Thermo Fisher Scientific, USA). The cultures were incubated in an incubator at 5% CO_2 and 37^{o}C. The media were replenished every 2-3 days and sub-cultured once it reached 70-80% confluency. Before the differentiation of NSC-34, the cultures were treated with trypsin-EDTA (Sigma-Aldrich, USA) to detach the cells from the flask. Approximately 6 x 10^4 NSC-34 cells per well were seeded into a 12 well-plate containing

the pre-treated coverslips for differentiation. The coverslips were pre-coated with poly-L-lysine (Sigma-Aldrich, USA). The differentiation media compose DMEM-F12, 1% of FBS, 1% of penicillin-streptomycin, 1% non-essential amino acid (NEAA; Sigma-Aldrich, USA), and trans-retinoic acid (RA; Merck, Germany), either 1µM and 10µM. Undifferentiated culture without RA was prepared as a control group.

Immunofluorescence staining with neuronal marker, Beta-III tubulin

NSC-34 cells were fixed in 4% of paraformaldehyde (PFA) on either day 1, day 3, day 5, or day 7 post-treatment to prepare for immunofluorescence (IF) staining. The cells were blocked and permeabilized in phosphate buffer saline (PBS; Thermo Fisher Scientific, USA) containing 0.3% Triton X-100 and 3% bovine serum albumin (BSA; Sigma-Aldrich, USA). Cells were stained with anti-Beta-III tubulin (Promega, USA) diluted in PBS containing 1% BSA (1:1000 dilution) for 24 hours at 4°C. After cells were washed with PBS, the cells were incubated with FITC-conjugated secondary antibody (1: 200 dilution; Jackson ImmunoResearch, USA) and 1% horse serum (Thermo Fisher Scientific, USA) in PBS. The cultures were then co-stained with DAPI (1:200; Thermo Fisher Scientific, USA) for 10 minutes. After removing DAPI, the cultures were stored in PBS.

Image acquisition

Images were captured using phase-contrast microscopy (Olympus, CKX31SF) and fluorescence microscopy (Leica, DM IL).

Results

Morphological differentiation of NSC-34 using different concentrations of retinoic acid

NSC-34 cells were treated under low serum conditions with 1µM or 10µM of retinoic acid (RA). The development of neurites in differentiated NSC-34 was observed for seven days. There were two types of cells present in the untreated sample; large cells with short neurites and small circular cells (Figure 1A). Upon treatment with 1µM of RA, neurites outgrowth appeared to be longer and more sprouts of neurite branches were observed (Figure 1B – E). Neurite outgrowth appeared to be more extensive when treated with a high RA concentration than cells under low RA concentration (1µM) (Figure 2A and Figure 2B).

Expression of Beta-III tubulin, a neuronal marker in differentiated NSC-34

To confirm the presence of neurons and to detect neurite outgrowth in the culture, NSC-34 cells were differentiated for 1, 3, 5, and 7 days in-vitro before being stained with anti-Beta-III tubulin antibody. Immunofluorescence analysis demonstrated that both undifferentiated and differentiated NSC-34 cells exhibited positive Beta-III tubulin expression (Figure 3 and Figure 4). The number of sprouting neurites increased, and the neurites became more elongated as the period of differentiation increases. Interestingly, when cells were differentiated with 10µM of RA for seven days in-vitro, Beta-III tubulin-positive cells appear to be larger, with large extension bodies protruding from the cell bodies.

Discussion

Loss of motor neurons is commonly found in progressive neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.[6,20,21] Animal models and primary neuronal cultures are commonly used to unravel the complex pathogenesis of these diseases. A hybrid cell line NSC-34, a fusion of murine motor neuron-enriched spinal cord with neuroblastoma cells, has been used in the physiological study of motor neurons and their degeneration [12,13,18].To induce the characteristic of motor neurons, the NSC-34 cells are subjected to differentiation process.[15,18,22] Differentiated NSC-34 exhibits unique neuron characteristics such as neurite

extension, and the expression of a neuronal marker, Beta-III tubulin.[12, 15,18, 23] The differentiation of cell lines often requires modification of the culture medium by culturing in a low serum condition with differentiation agents such as retinoic acid (RA). RA has the property to decelerate proliferation, promote neurite outgrowth and differentiate NSC-34 cell lines into motor neuron-like cells.[15,17]

Many studies have reported the use of 1µM of RA to differentiate NSC-34 cells.[15,18] However, 10µM of RA is an ideal concentration commonly used to differentiate a different cell line, called human neuroblastoma, SH-SY5Y, into mature neurons.[24,25] Therefore, this study aimed to optimize the differentiation protocol of motor neuron-like cell line, NSC-34, by comparing two different concentrations of RA, 1 µM and 10 µM. Results from phase-contrast microscopy demonstrate that at 1µM of RA, development of neurites was observed starting from one day post-differentiation. The length of neurites increases, and more neurite protrusions were observed as the period of differentiation increases. Similarly, when 10µM of RA was used, the NSC-34 cells developed more extended neurites.

Another indicator to identify neurons is through the expression of a neuronal marker, Beta-III tubulin. Beta-III tubulin is a cytoskeletal protein that is commonly used for neuronal identification.[15,17] Results show positive-Beta-III tubulin staining in undifferentiated and differentiated NSC-34 cells at 1, 3, 5, and 7 days post-differentiation. Beta-III tubulin expression in undifferentiated cells is expected as the protein is essential for neuronal functions, including neurite outgrowth.[26,27] Interestingly, when 10µM of RA was used, the length and number of the neurites appeared to be increased. This observation may indicate rapid neuronal development to become mature neurons.

Conclusion

Retinoic acid (RA) treatment at 10µM concentration promotes an efficient differentiation of NSC-34 cells into mature neurons compared to 1µM. Extensive neurite sprouting and longer neurite length indicate better neuronal maturation when using 10µM RA concentration. The study supports the use of 10µM RA treatment for NSC-34 differentiation into motor neuron-like cells, which can be helpful to generate a suitable model for electrophysiology studies and studies involving axonal transport.

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Conflict of Interest

The authors declare no conflict of interest in the publication of this article.

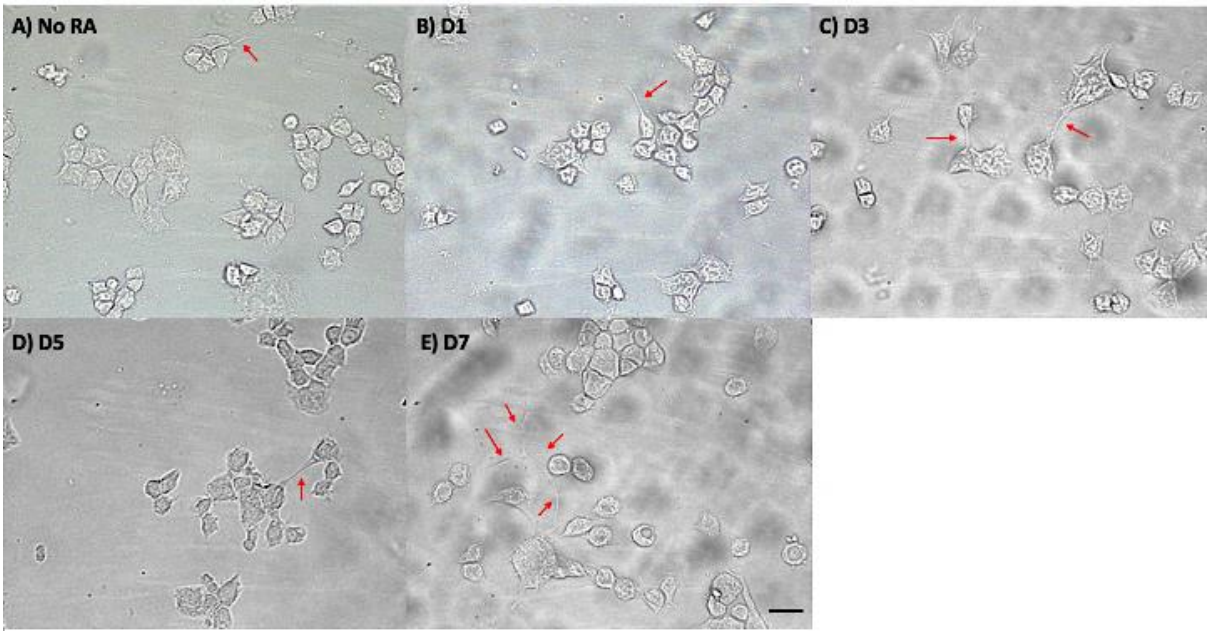

Figure 1: Morphological differentiation of NSC-34 cells at 1µM of retinoic acid. Cells were observed under phase-contrast microscopy at 20x magnification. Undifferentiated cells served as a negative control (A). For the treated group, cells were differentiated for 1(B), 3 (C), 5(D), and 7 (E) days in-vitro. The red arrow represents neurites. Scale bar represents 50µm. Abbreviations: Day (D); Retinoic acid (RA).

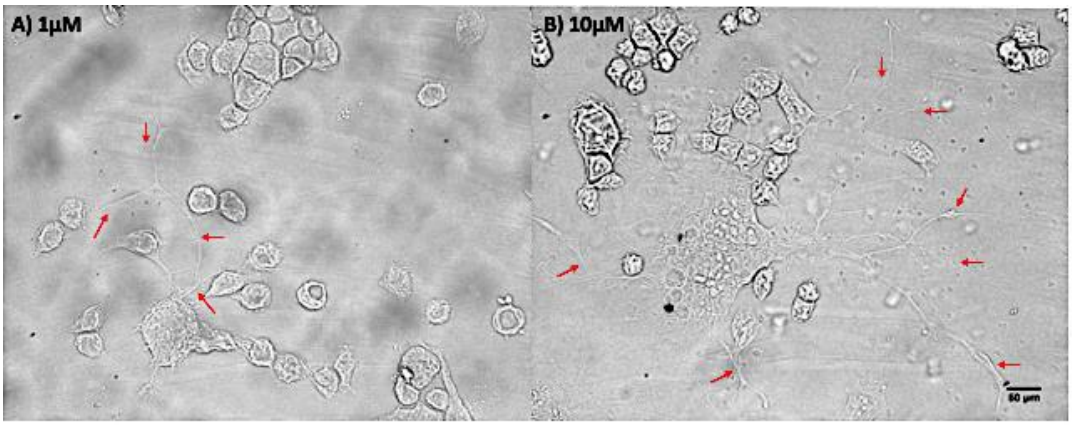

Figure 2: Comparison of differentiated NSC-34 cells when 1µM and 10µM of retinoic acid (RA) were added. Cells were observed under phase-contrast microscopy at 20x magnification. Cells were differentiated for seven days in-vitro. The red arrow represents neurites. Scale bar represents 50µm.

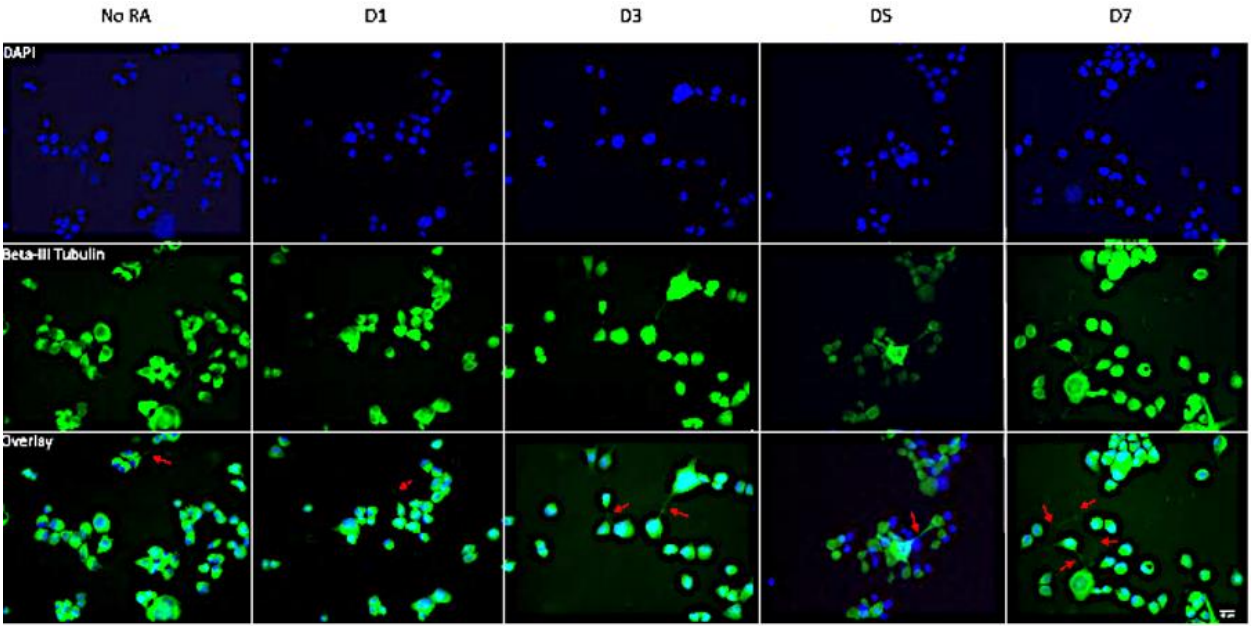

Figure 3: Observation of differentiated cells stained with Beta-III tubulin.

A) 1µM of retinoic acid was added to the cells. Cells were observed under fluorescence microscopy on day 1, 3, 5, and 7. Cells were observed under fluorescence microscopy on day seven. Cells were fixed in 4% PFA before being stained with Beta-III tubulin (green) and DAPI (blue). The cells were observed under 40x magnifications. Scale bar represents 20µm. The red arrow represents neurite. Abbreviation: Retinoic acid (RA).

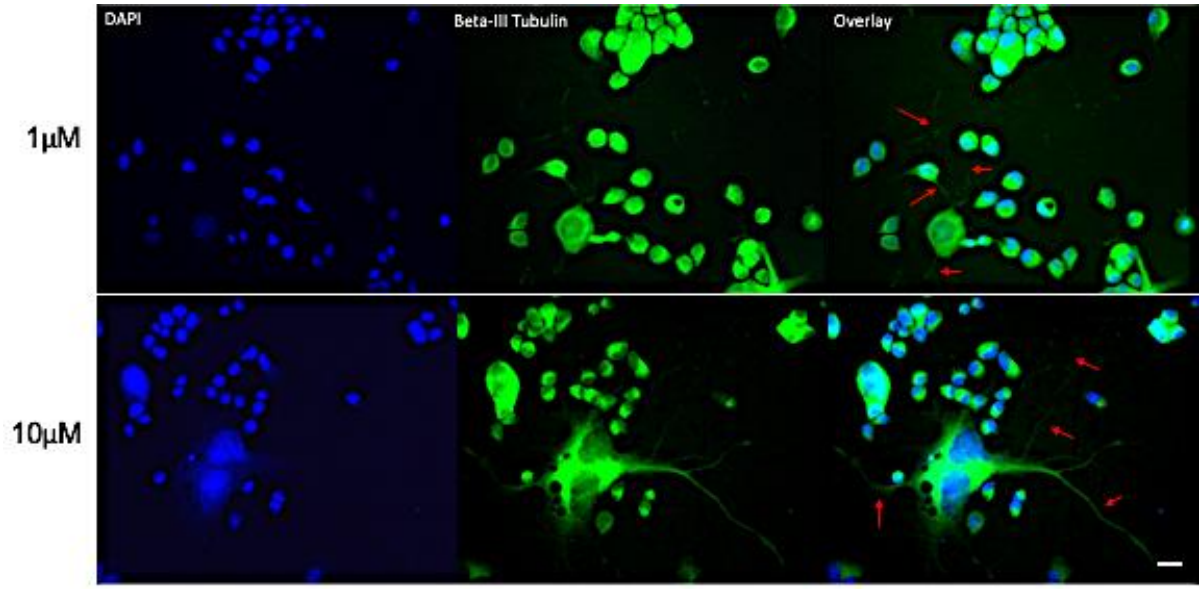

Figure 4: Detection of Beta-III tubulin expression in cells differentiated with 1µM or 10µM of retinoic acid. Cells were observed under fluorescence microscopy on day seven. Cells were fixed in 4% PFA before being stained with Beta-III tubulin (green) and DAPI (blue). The cells were observed under 40x magnifications. Scale bar represents 20µm. The red arrow represents neurite. Abbreviation: Retinoic acid (RA).

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