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Prospective stem cell lines as *in vitro* neurodegenerative disease models for natural product research

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ABSTRACT: The use of *in vitro* model for screening pharmacological compounds or natural products has gained global interest. The choice of cells to be manipulated plays a vital role in coming up with the best-suited model for specific diseases, including neurodegenerative diseases (ND). A good *in vitro* ND model should provide appropriate morphological and molecular features that mimic ND conditions where it can be used to screen potential properties of natural products in addition to unravelling the molecular mechanisms of ND. In this mini review, we intend to demonstrate two prospective stem cell lines as the potential cell source for *in vitro* ND model and compare them to the commonly used cells. The common source of cells that have been used as the *in vitro* ND models is discussed before going into details talking about the two prospective stem cell lines.

Keywords: Neurodegenerative diseases; Natural products; Stem cell; *In vitro* ND models;

1. INTRODUCTION

Neurodegenerative diseases (ND) is the term used for various conditions, primarily marked by the loss of nerve cells, resulting in functional impairment of neurons. The progressive degeneration and/or death of nerve cells and the limited ability of the brain to self-repair subjects ND to be incurable. As degenerative diseases are often linked to differentiation stimulation, many therapeutic strategies for neurodegenerative diseases have been studied with emphasis on the regulation of cell differentiation [1]. Although the neurological mechanisms of action are still not fully understood, it was previously reported that the cell differentiation, survival, protection and regeneration were shown to be influenced by the neurotrophins and growth factors, especially the nerve growth factors (NGF) and brain-derived neurotrophic factors (BDNF) [2].

Herbal medicine research has gained increasing interest globally for their therapeutic potentials. Extensive practise of herbal medicine provides promising approaches to current therapies for ND. Researchers revealed that the presence of phytochemicals in herbal extracts including total alkaloids, phenolics, flavonoids, tannins and terpenic acids, exhibit promising anti-inflammatory, antioxidant, neuroregenerative and neuroprotective properties, as well as anticholinesterase and neurotrophic-like activities with lesser adverse effect. Furthermore, some of them promote cell survival and improve cognitive functions by directly regulating amyloidogenesis and apoptotic signalling pathways. Some promising natural products that may have therapeutic potentials for the treatment of ND and the cell lines used as the *in vitro* models are summarised in Table 1.

Hence, screening of pharmacological compounds or herbal extracts for their neuro-pharmacology properties would provide the means to gain knowledge on the morphological features and molecular mechanisms of ND in addition to gaining insights towards the development of various types of treatment for these diseases. Initial screening of these properties could be first done through the use of appropriate *in vitro* ND model, thus creating the best suited *in vitro* models for ND in cell culture is invaluable and essential. Table 1 shows the cell lines that have been used as ND models to screen the neurotherapeutic potential of phytochemicals from natural products. There are several approaches to generating *in vitro* models for neurodegenerative diseases based on aetiology and mechanisms involved in the diseases. In attempting to mimic the same phenomenon occurring in animal models of ND, the synthetic compounds/toxins which possess neurotoxicity activity are introduced to cultured cells to induce cell injury and activate the apoptotic signalling pathways that are correlated with the disease of interest. The most commonly used synthetic compounds/toxins are (1) β-amyloid, (2) glutamate, (3) 6-hydroxydopamine (6-OHDA), (4) 1-methyl-4-phenylpyridine (MPP+), (5) rotenone, and (6) hydrogen peroxide (Table 2). Another approach is by genetic modification such as (1) Presenilin 1 (PSEN1); (2) Presenilin 2 (PSEN2) [3]; and (3) Amyloid precursor protein genes [4]. Besides the choice of a neurotoxic agent used, the source for neural/neuronal cells also plays an important role in deciding the appropriate *in vitro* model to use.

In this research note, we aim to demonstrate the suitability of using stem cell lines, namely the mouse embryonic (46C) and rat full-term amniotic fluid (R3) stem cell lines, as the prospective source of neuronal cells in creating the models that could later be used to aim for screening neuro-pharmacology properties of an extract or natural compound, as opposed to the commonly used cell lines. In doing so, the features and properties of the commonly used cell lines as the cell source for developing *in vitro* model for ND is discussed and compared to that of 46C and R3 lines.

2. COMMONLY USED CELL LINES FOR *IN VITRO* ND MODEL

The commonly available *in vitro* models that are widely used for ND studies come from three cell sources: (1) Human neuroblastoma (SH-SY5Y); (2) immortal rat

hippocampal; and (3) Induced pluripotent stem cell lines.

Table 1: Neurotherapeutic potential of phytochemicals from natural products

Plant source	Phytochemical	Pharmacological effects	Medicinal use	Ref(s)
Ginkgo biloba	Ginkgolide B	ROS scavenger inhibits NF-κB activation and PI3K/Akt signalling, inhibition of apoptotic protein expression, activates Wnt/β-catenin signalling pathway. ***In vitro* model used:** Rat primary cerebellar neuron cells; neural stem cells of the postnatal mammalian subventricular zone; rat primary hippocampal neuronal cells; human neuroblastoma (SH-SY5Y) cells.	Neuroprotection, neurodegeneration disease, antioxidant	[5-9]
Vitis vinifera, *Vaccinium macrocarpon*	Resveratrol	Antioxidant promotes the decomposition and clearance of intracellular Aβ aggregates, inhibits glial cell activation, activates SIRT1 expression, inhibits iNOS, COX-2, NF-κB activation. ***In vitro* model used:** Rat pheochromocytoma (PC12) cells; rat primary cortical mixed glial cells; human neuroblastoma (SH-SY5Y) cells; rat primary neuronal cells.	Neuroprotection, neurodegeneration disease, antioxidant	[10-21]
Huperzia serrata	Huperzine A	* AChE inhibitor, reduces protein levels of IL-1β and TNF-α, inhibits NF-κB activation, and increases BDNF and NGF level. ***In vitro* model used:** Rat pheochromocytoma (PC12) cells; rat primary cortical neuronal cells.	Neuroprotection, neurodegeneration disease, antioxidant, anti-inflammation	[22-26]
Rhodiolia Rosea	Salidroside	Inhibits Aβ-induced oxidative stress through activation PI3K/Akt signalling, suppresses the expression of MAPK and JNK, ROS scavenger. ***In vitro* model used:** Human neuroblastoma (SH-SY5Y) cells; murine BV-2 microglial cells; rat pheochromocytoma (PC12) cells; rat primary hippocampal neuronal cells.	Neuroprotection, neurodegeneration disease, antioxidant	[27-34]
Curcuma longa	Curcumin	Increases BDNF level, ROS scavenger, prevents Aβ fibrils, inhibits COX-2 and NF-κB activation activates PKC/ERK-mediated CREB regulation. ***In vitro* model used:** Rat pheochromocytoma (PC12) cells; human neuroblastoma (SH-SY5Y) cells	Antioxidant, anti-inflammation, neurogenesis, neuritogenesis	[35-41]

* AChE: Acetylcholinesterase

Table 2: The most common synthetic compoundsm/toxins used in ND *in vitro* models

Common synthetic compounds/toxins	Disease model *	References
β-amyloid	AD	[4,42-44]
Glutamate	AD	[45]
6-hydroxydopamine (6-OHDA)	PD	[46]
1-methyl-4-phenylpyridine (MPP+)	PD	[46]
Rotenone	PD	[47]
Hydrogen peroxide	ND associated with oxidative stress, especially PD and AD	[13,27,34,48-51]

***** AD = Alzheimer 's disease; PD = Parkinson's Disease

Human neuroblastoma cell lines (SH-SY5Y) has been used widely in neuroscience research. SH-SY5Y cells are a subclone of the parental neuroblastoma SK-N-SH cell lines that were derived from bone marrow biopsies, consists of neuroblast-like and epithelial-like cells [52]. SH-SY5Y cells consist 47 chromosomes, making it a stable karyotype and able to differentiate into mature neurons by induction with retinoic acid (RA) or neurotrophins such as brain-derived neurotrophic factor (BDNF) [53]. SH-SY5Y has been used to establish *in vitro* model for Alzheimer Disease (AD) and Parkinson Disease (PD), as well as other neurodegenerative diseases. SH-SY5Y can express functional neuronal subtypes when differentiate, as such into synaptic structures, functional axonal vesicle transport and express neuronal markers NeuN, class III β-tubulin and synaptic vesicle protein 2 (SV2) [54]. A study conducted by Constantinescu and coworkers showed that SH-SY5Y was successfully induced into dopaminergic neurons by RA at a concentration of 10 µM for a few days [55]. This finding was in a good agreement with another study that reported the presence of tyrosine hydroxylase (TH) (a marker for dopaminergic neurons) gene expression post-differentiation [56].

Besides RA, the SH-SY5Y cell line also has been used as a model to study PD when exposed to rotenone, a specific inhibitor of mitochondrial complex I that can cause mitochondria impairment leading to increasing in oxidative stress. The administration of rotenone has been observed to induce cell apoptosis and accumulation of reactive oxidative species (ROS) [49,57,58]. Another study also showed that treatment of SH-SY5Y with rotenone might involve mitochondria- and endoplasmic reticulum-dependent caspase pathways, promoting cell death in concentration- and time-dependent manner [57]. Rotenone-induced SH-SY5Y neurons were also used to evaluate the protective effect of antioxidant property of the phenolic compound. The phenolic compound was found to exert protective effects against the cytotoxicity of rotenone and increased the cellular GSH contents [58].

Interestingly, Aβ-induced oxidative stress in SH-SY5Y also elicits the same mechanisms [28] as observed in rotenone-induced cell damage. Using this model, treatment with antioxidant salidroside was found to restore cell survival and enhance the expression of antioxidant genes [28]. In another study, the SH-SY5Y cell line was used to test the effect of asiatic acid of *Centella asiatica* in inhibiting glutamate-induced SH-SY5Y from undergoing apoptosis and reducing ROS activity when compared to the untreated cells [45]. These exciting features of a cytotoxic-induced SH-SY5Y cell line may benefit in finding the treatment regime for PD and AD. However, this model does not mimic the real scenario in human ND due to different cell signalling pathways by cancer genes [59].

Due to the high demand for using normal brain cells *in vitro*, that express specific neuronal subtypes, immortal rat hippocampal cell lines were studied. These cells were immortalised using retroviral-mediated oncogene transduction Simian virus 40 large tumour antigens [60].

The cells have two distinct properties which are: (1) restricted proliferation; and (2) ability to differentiate after completion of neuronal division. Immortal rat hippocampal cell lines exhibit neural subtype markers such as glial fibrillary acid protein (GFAP), Neurofilament protein (NFP) and synthesise neurotrophic factors [60]. Since the cells were originated from the hippocampus, which is responsible for cognitive and memory function, these cell lines are suitable for AD model. These cells have been used to evaluate the neuroprotection activity of herbal medicines intended for the treatment of ND. A study showed that four traditional Oriental medicinal herbs protect the rat hippocampal cells from glutamate-induced toxicity by reducing the ROS production and increasing cell viability [61]. Although these cell lines provide a promising approach to evaluate the neuroprotection and neuroregeneration potential of herbal medicines, its action is limited to the animal AD, and it cannot induce the full range of deficits seen in human with AD due to lack of receptor for human Aβ peptides [59].

The reprogramming of the somatic cells to induced pluripotent stem cells (iPSCs) to model ND pathogenesis *in vitro* has been established and accepted worldwide. The recent review article discusses the potential use of PD-specific iPSCs to model the pathogenesis of the disease due to genetic variants carried in iPSCs [62]. In a comparison study between the ability of iPSC and embryonic stem cells (ESCs) to differentiate into dopaminergic neurons revealed that iPSCs show higher differentiation ability compared to ESCs [63]. These findings show that iPSCs can provide a promising source for dopaminergic neurons in modelling PD *in vitro*. A recent study shows that disease-/patient-specific iPSC-derived neurons can provide a better understanding of the drug screening. A study conducted by Cooper *et al*. showed neurons from PD patients that carrying mutations in the *PINK1* or *LRRK2* genes exhibited oxidative stress and mitochondria impairment [64]. In addition, the AD model was also established from primary human fibroblast cells collected from familial Alzheimer's disease (FAD) patient and was reprogrammed into iPSC. From this iPSC, two AD *in vitro* models were established with different clones using Presenilin 1 (PSEN1); (2) Presenilin 2(PSEN2) mutations. The genetic modified-iPSCs were then undergoing neural differentiation to model *in vitro* AD pathogenesis [3]. However, this model is time-consuming with a high risk of mutations [59].

3. PROSPECTIVE STEM CELL LINES AS AN *IN VITRO* ND MODEL

Unlike SH-SY5Y cells and immortal rat hippocampal cells, stem cells are unique cells with the capacity to self-renew [65] and can be differentiated into a vast variety of cell types including neurons *in vitro* [66]. Several studies have shown the beneficial effect of stem cells in degenerative diseases due to their capacity to differentiate into any types of cells and also their ability to secrete trophic factors that can reverse the damaged tissues [67]. Furthermore, stem cells become precious tools to establish *in vitro* model of ND and to study the therapeutic strategies owing to their capability to differentiate into any types of cells. To expand the choice of stem cell use as the prospect cells for establishing ND in vitro models, we are exploring and manipulating the properties of mouse embryonic stem cell (mESCs), 46C, and rat amniotic fluid stem cell (AFSCs), R3, lines. 46C is a mESCs transgenic line that carries a green fluorescent protein (GFP) knocked-in into the open reading frame of a transcription factor gene, *Sox1*, a marker for neural precursor cells (NPCs) [68]. Meanwhile, R3 is our in-house established AFSCs generated from rat full-term amniotic fluid. Both 46C and R3 have been shown to have the ability to differentiate into neurons [69-72].

Mouse ESCs can be acquired from (1) dissociated morulae [72]; (2) intact blastocysts [73]; and the inner cell mass (ICM). Generally, mESCs were isolated from E3.5 ICM of the mouse blastocyst [74] into tissue culture and propagating them in the presence of leukaemia inhibitory factor (LIF) [75] or murine embryonic fibroblast (MEF) feeder layer cells [76]. ESCs are characterised by their ability to proliferate indefinitely *in vitro* while maintaining their pluripotency properties

[73]. ESCs can differentiate into the derivatives of the three primary germ layers, which are: (1) mesoderm; (2) endoderm; and (3) ectoderm [77-79].

Three fundamental transcription factors are involved in maintaining the pluripotency and self-renewal of ESCs, namely: (1) *Oct4*, belongs to POU family transcription; (2) *Sox2*, a member of SRY-related HMG box (Sox) family; and (3) *Nanog*, from the homeobox DNA binding family [80-83]. *Oct4* gene is highly expressed in the ICM of the blastocyst, during early embryogenesis [84], as well as in pluripotent cells and it plays a crucial role in the cell fate determination [85]. Another transcription factor, *Sox2* plays a crucial role in embryonic development and is also associated with pluripotency of ESCs [80]. *Sox2* is also involved in the proliferation of precursor cells and differentiation of neuronal specific subtypes during the development of the central nervous system (CNS) [86]. Similar to *Oct4* and *Sox2*, Nanog is also essential in the maintenance of pluripotency and self-renewal of ESCs [87,88]. In addition, Silva *et al.* and Mitsui *et al.* reported that N*anog* is a key factor in the development of ICM and germ cells, suggesting its involvement in maintaining the pluripotency and self-renewal of the cells [87,89]. These three transcriptional genes work in the dependence manner in order to maintain self-renewal property while inhibiting the differentiation of the ESCs by co-operatively bind to their promoter and forming interrelated auto-regulatory loop [73,81,82].

On the other hand, amniotic fluid consists of numerous cell types derived from a growing foetus which ultimately differentiated into various cell types such as adipose, muscle, blood and neural lineage [90]. Currently, research on stem cells derived from amniotic fluid have been extensively studied. AFSCs can be obtained during the second and third trimester of pregnancy or directly after birth in human; and in the second or third week of pregnancy in mice and rats [71,90,91]. Two major populations of stem cells were found in amniotic fluids as reported by Cananzi and co-workers, namely; (1) amniotic fluid mesenchymal stem cells and amniotic fluid stem cells (AFSC) [92]. We have successfully isolated and characterised AFSC in rats [71]. We categorised these cells as broadly multipotent stem cell based on the presence of the surface antigen, c-kit (CD117, a type III tyrosine kinase receptor of stem cell), which distinguishes it from mesenchymal stem cells [71]. The cells were observed to express Oct4 and Nanog. Our results were also supported by the study conducted by De Coppi and co-workers on the ability of rat AFSC to differentiate into the three primary germ layers: (1) mesoderm; (2) endoderm; and (3) ectoderm [71,90]

Both 46C and R3 can be directly induced to differentiate into neural lineage through the (1) formation of multicellular aggregates, embryoid bodies, EBs, by 4-/4+ protocol [69], with the addition of *all-trans* retinoic acid (ATRA) as the neural inducer and also *via* (2) monoculture adherence protocol [93]. The abilities of mESCs and rat AFSCs to differentiate into functional neuronal and neuronal supporting cell subtypes have been reported previously [69,71]. These discoveries were in good agreement with our study with 46C and R3 (Figure 1 and 2). Both cell lines were able to generate good quality EBs (Figure 1) and differentiate into immature and mature neurons, as well as glial cells (Figure 2). Their neurogenic potential was observed to be similar to SH-SY5Y cells and immortalised rat hippocampal cell lines based on the marker expression profile (Table 3). All of these cell lines express markers for post-mitotic and mature neurons, as well as glial cells.

The neurogenic properties of these cell lines have prompted us to test the suitability of their generated neurons as a tool to establish an *in vitro* ND model, as a prospective model for natural product research or at least for initial screening of neurotherapeutic potential of natural products. The in vitro model was established by treating R3-and 46C-derived neurons with hydrogen peroxide (H_2O_2).

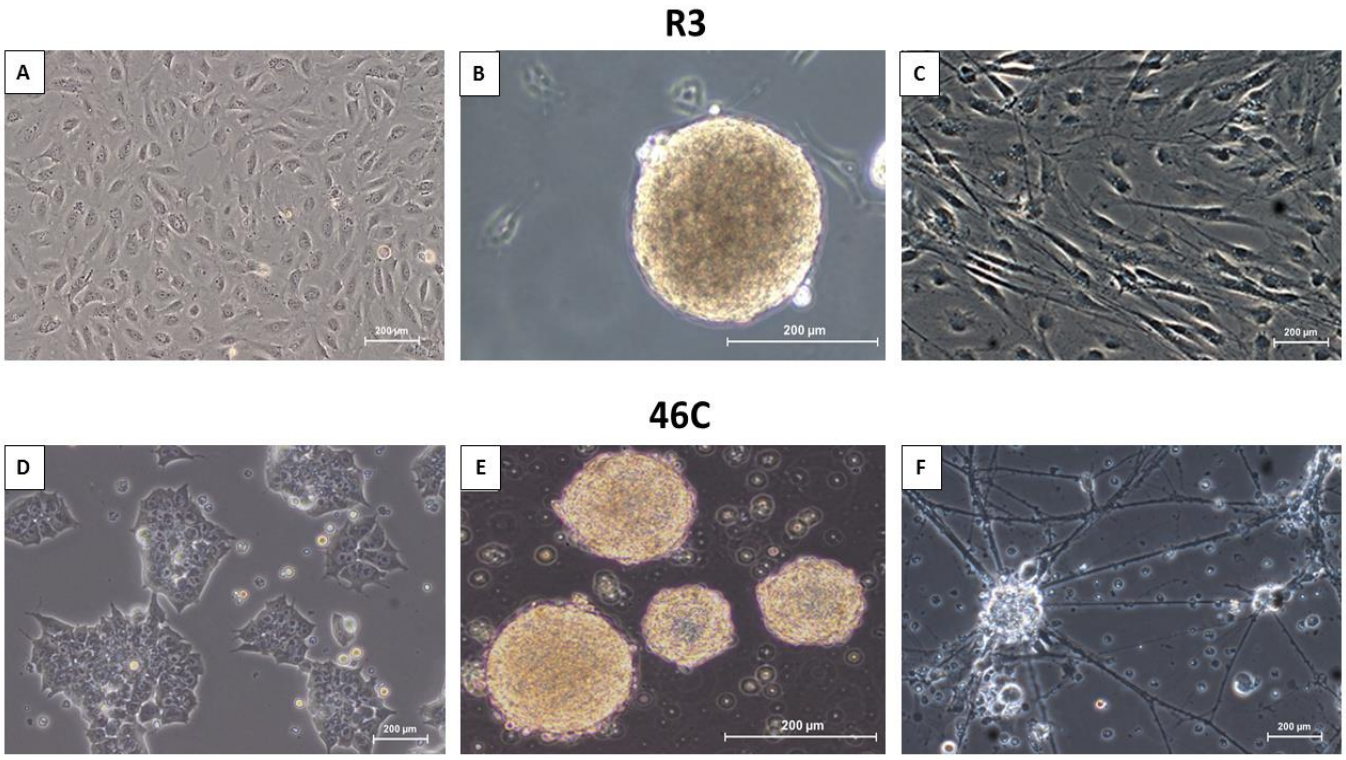


Figure 1: Differentiation of 46C and R3 after neural induction using 4-/4+ protocol. **(A)** Undifferentiated R3; **(B)** Day 4 R3-derived EBs; **(C)** Day 6 post-plating R3-derived neurons onto gelatin-coated plate; **(D)** Undifferentiated 46C; **(E)** Day 4 46C-derived EBs; and **(F)** Day 8 post-plating 46C-derived neurons onto PDL/Laminin-coated plate. The scale bars represent 200 µm for micrographs.

H_2O_2 was chosen for the fact that it has been used as the most common toxin to induce oxidative stress condition which is the general hallmark in ND conditions, particularly in PD and AD [43-45,50-54]. Based on cytotoxicity assay using MTT (Figure 3A), high concentrations of H2O2 (1500 µM for 46C and 2000 µM for R3) were chosen. Our data showed a significant increase in ROS activity when the neurons were treated with H2O2 at these concentrations (Figure 3). Our results indicate a prospective success of establishing ND models in vitro by stimulating the production of reactive oxygen species (ROS). These findings clearly demonstrate the potential of these stem cell lines to provide good sources for an ideal in vitro model that could be mimicking ND phenomenon. Unlike SH-SY5Y, which is a cancerous cell line, these stem cell lines may provide more reliable sources of neurons as they represent a normal condition before the pathogenicity of ND develops. The effects of using more toxins such as those described in Table 2 should be carried out in the effort to characterise new in vitro models in future studies. Nevertheless, more in-depth investigations looking at different perspectives of an in vitro ND model are required before one can use it as the model. In a nutshell, these cell lines may be efficiently manipulated to establish models that would be applicable for initial screening of drugs or neurotherapeutic molecules of natural products, and fundamental studies before in-vivo pre-clinical studies to take part.

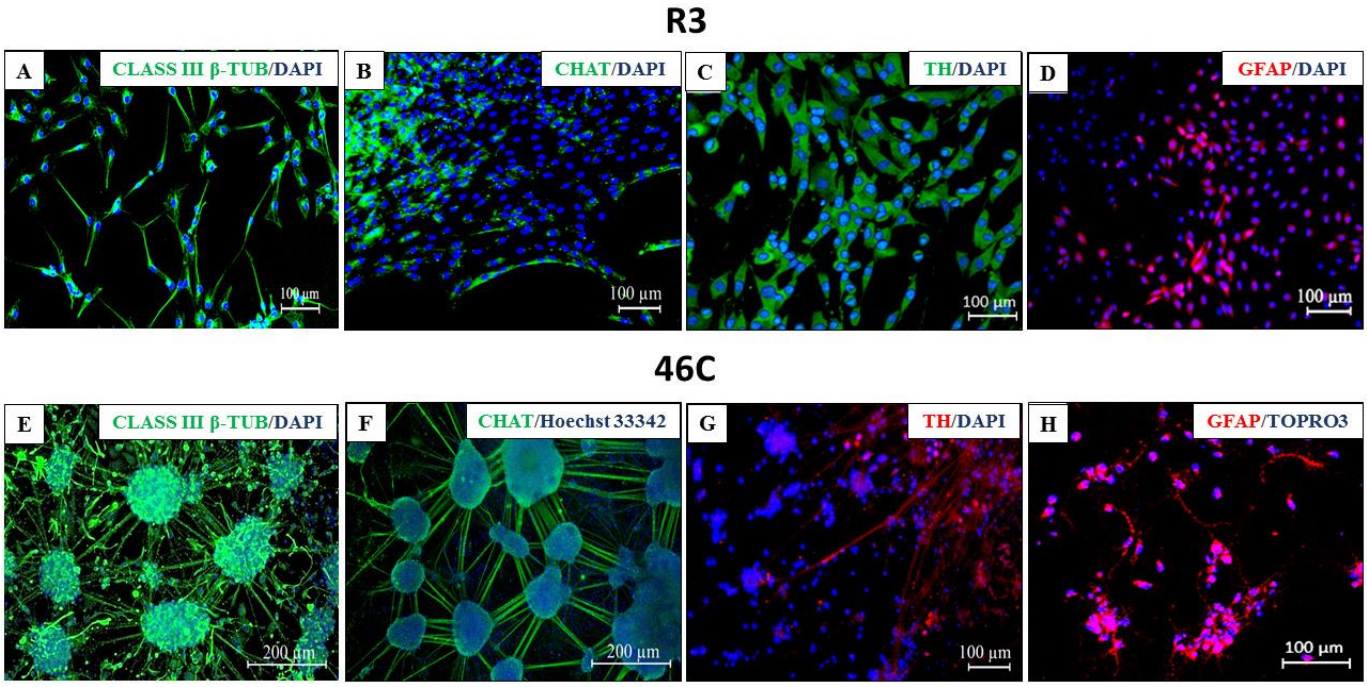


Figure 2: Immunostaining of neural specific markers after neural differentiation of R3 and 46C from both monolayer adherent and 4-/4+ protocols. **(A)** and **(E)** are immunostaining of class III ß-tubulin (post-mitotic neurons); **(B)** and **(F)** are immunostaining of choline acetyltransferase, CHAT, (cholinergic neurons), **(C)** and **(G)** are expression of tyrosine hydroxylase, TH, (dopaminergic neurons), and **(D)** and **(H)** immunostaining of glial fibrillary acidic protein, GFAP, (astrocytes) in R3 and 46C-derived neurons, respectively. Nuclei were counterstained with DAPI, TOPRO3 or Hoechst 33342 (blue). The neural specific markers highly indicate that adherent monoculture and 4-/4+ protocol have efficiently generated not only neuronal cells but also neuron supporting cells (glial cells) in R3 and 46C, respectively. R3-derived cells were from D12 **(A)** and D8 **(B, C and D)** neural cells plated onto gelatin-coated plates and 46C-derived cells were from D4 **(E and F)** neural cells post-plated onto PDL/Laminin-coated plates and D14 **(G and H)** post-plated onto gelatin-coated plates. Class III ß-tubulin, TH and GFAP positive cells were obtained through monolayer adherent method; while ChAT-positive cells were obtained via 4-/4+ method. The scale bars represent 100 and 200 µm for micrographs.

Table 3: Comparison of marker expression profiles of SH-SY5Y and rat immortalised hippocampal cells to 46C and R3

Marker expression / Cell lines	Post-mitotic neurons	Mature neurons	Glial Cells
Human neuroblastoma (SH-SY5Y)	NeuN and Class III β-tubulin [20]	Tyrosine hydroxylase (TH) [56] Synaptic vesicle protein 2 (SV2) [60]	-
Rat immortalised hippocampal cells	-	Neurofilament protein (NFP) [60]	Glial fibrillary acid protein (GFAP) [60]
Embryonic stem cell (46C)	Class III ß-tubulin	Choline acetyltransferase (ChAT) Tyrosine hydroxylase (TH)	Glial fibrillary acidic protein (GFAP)
Rat amniotic fluid cell (R3)	Class III ß-tubulin	Choline acetyltransferase (ChAT) Tyrosine hydroxylase (TH)	Glial fibrillary acidic protein (GFAP)

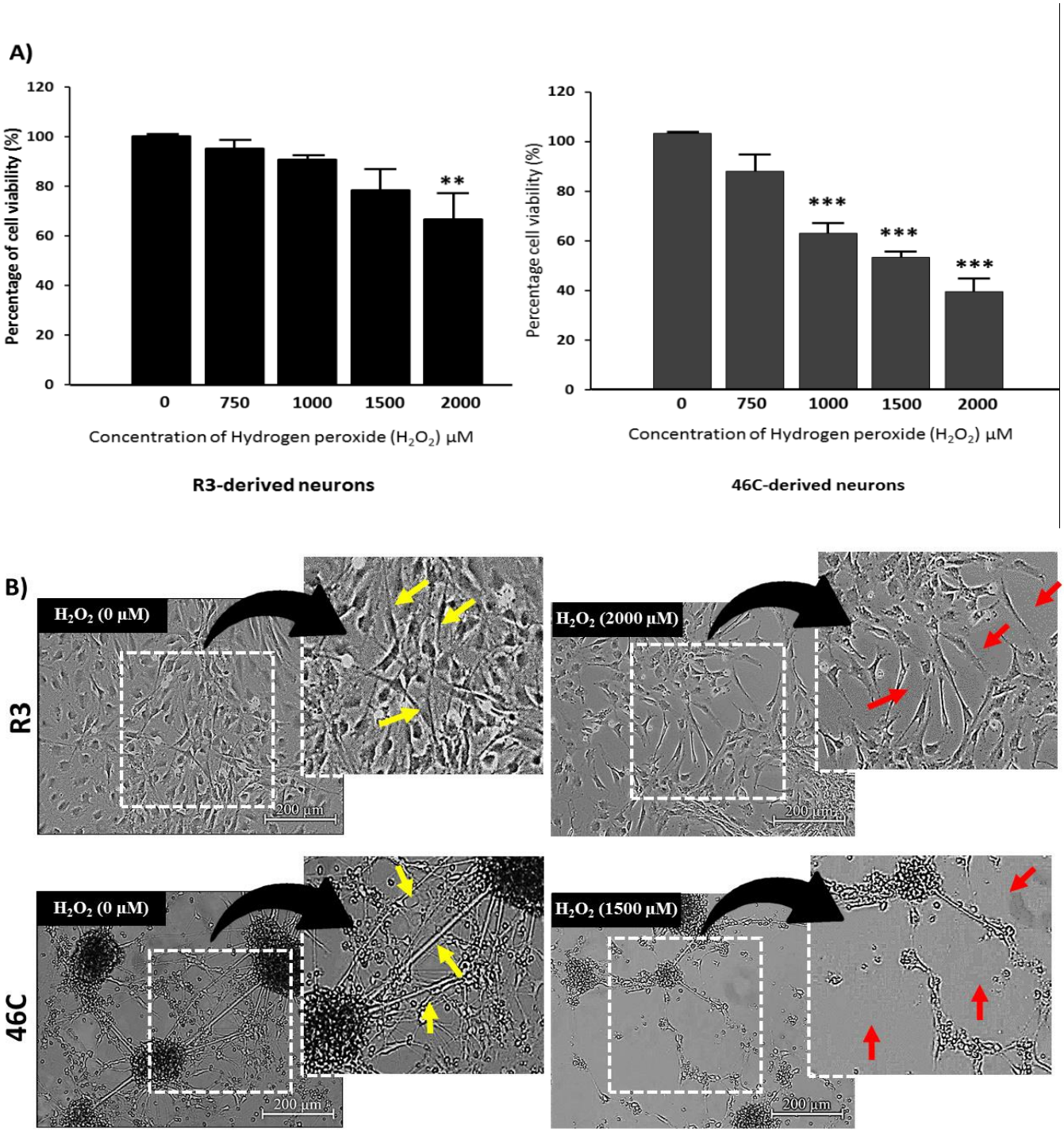
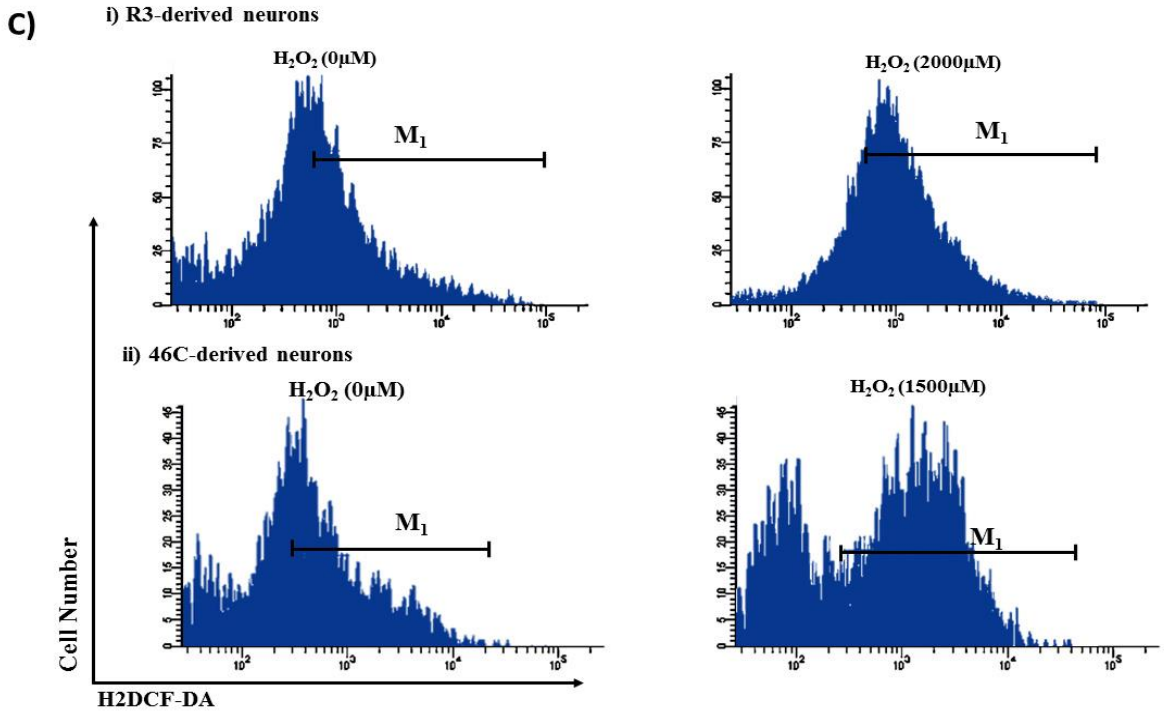


Figure 3: (A) The effect of different concentrations of H_2O_2 on R3- and 46C-derived neurons expressed as the percentage of cell viability by MTT assay. Errors bars (I) indicate ± standard error of mean from 3 independent experiments, each with 3 technical replicates (n=3) where *** indicates p<0.001 and ** indicates p<0.01 (One way ANOVA: Tukey's test multiple comparisons) compared to untreated neurons. **(B)** Morphology of untreated (0 µM H_2O_2) with neurite projections (indicated by yellow arrows); 2000 and 1500 µM H_2O_2 -treated day 8 post-plated R3-derived neurons and day 4 post-plated 46C-derived neurons, respectively, after 24 h treatment. The neurons were damaged upon treatment with H_2O_2. Neurite projections damaged in neuronal culture treated with H_2O_2 (indicated by red arrows). The scale bars represent 200 µm for micrographs. ROS activity was assessed by H2DCF-DA assay, as depicted in the histogram **(C, *next page*)** and percentage of ROS generation versus neurons graph **(D, *next page*)**. **(C, *next page*)** Intracellular ROS generation within the cells labelled with 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF- DA) was measured by flow cytometry. Intracellular ROS oxidized H2DCF-DA into fluorescent 2', 7'- dichlorodihydrofluorescein diacetate (DCF). ***Continued on next page.***



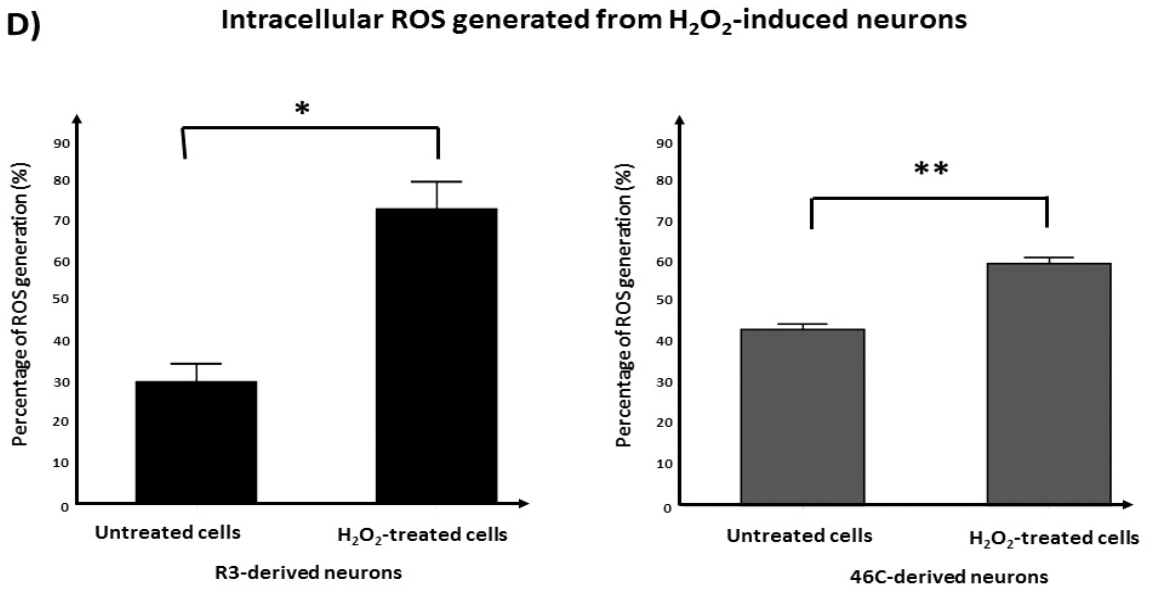


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Figure 3: In our study, the neurons were pre-incubated with H_2O_2 at the concentrations of 0 µM which served as control, 1500 and 2000 µM (46C- and R3-derived neurons, respectively) for 24 h at 37°C, then incubated with DCFH-DA for 45 min at 37°C. The X-axis of H2DCF-DA histogram represents the H2DCF-DA intensity, while the Y-axis represents the cell counts in corresponding DCF fluorescence intensity. The percentage of ROS release within the cell population was shown by the shift of histogram peak to M_1 zones, defined as positive cells. Our results show that the peaks of H_2O_2 -treated neurons at a concentration of 1500 µm and 2000 µM, were gradually shifted to the right of M_1 zones compared to the untreated neurons (0 µM H_2O_2), reflecting an increased level of intracellular ROS within the cell population **(D)**. 2000 and 1500 µM of H_2O_2 induced a significant increase in ROS level of R3- and 46C -derived neurons, respectively, as compared to untreated neurons (control). The values represent the percentage of generated ROS. The error bars indicate mean ± Standard deviation (SD) from 1 independent experiment with 3 technical replicates (n=3) for R3-derived neurons and 46C-derived neurons, where ** indicates $p<0.01$ and * indicates $p<0.05$ (Student's *t*-test).

4. CONCLUSIONS AND FUTURE PROSPECTS

Neurons derived from animal stem cell lines may serve as high prospective cells to be utilised in the establishment of an *in vitro* ND model. They may provide better models for initial screening of neuro-pharmacology properties of drugs or natural products from a more reliable source that represents a normal condition compared to using cancerous cell line such as the neuroblastoma cell line. Although these two animal stem cell lines retained the capacity for neuronal and glial differentiation and can be grown for an extended period, data on human stem cells would be more appropriate as it is more reflective of human scenario and would be more suitable for medical applications. Thus, further studies using human stem cells should be looked into in the future.

Supplementary Materials: The following are available online at https://neuroscirn.org/ojs/index.php/nrnotes/article/-view/25. Method S1: Propagation of 46C mouse embryonic stem (mES) cell and Rat amniotic fluid stem cell (R3); Method S2. Neural differentiation assay; Method S2a. Neural differentiation assay trough EBs formation; Method S2b. Neural differentiation assay through adherent monoculture; Method S3. Immunocytochemistry; Method S4. Analysis of intercellular reactive oxygen species (ROS); Table S1. List of antibodies used for immunocytochemistry.

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Author Contributions: All authors read and approved the final manuscript. NI and NN designed the experiments; NI performed the experiments; NN, KHL, RR, ZH contributed reagents/analytical tools; NI and NN analysed data and drafted the article.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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