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Effects of Collagen Coating, Fetal Bovine Serum Concentration, Differentiation Agents, and Neurotoxin Application on In Vitro Modeling of Parkinson's Disease Using SH-SY5Y Cell Culture

SH-SY5Y Hücrelerinin Kültürlenmesinde Kollajen Kaplama, Fetal Sığır Serum Konsantrasyonu, Diferansiyasyon Ajanları ve Nörotoksin Uygulamasının Parkinson Hastalığının İn vitro Modellemesine Etkileri

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ABSTRACT

Objective: This study aims to optimize SH-SY5Y culture conditions to develop precise in vitro disease models for Parkinson's disease (PD) research. It seeks to investigate the effects of various factors such as collagen coating, fetal bovine serum (FBS) concentration, differentiation agents, and neurotoxin treatments on cellular behavior and disease modeling.

Materials and Methods: The human neuroblastoma SH-SY5Y cell line was cultured in DMEM/F12 supplemented with heat-inactivated FBS, penicillin-streptomycin, and L-glutamine. Collagen coating was applied to assess its impact on cell differentiation, while the ideal cell density and serum ratio for generating neurite-like cells were determined through experimentation. The MTT assay was employed to evaluate the cytotoxic effects of paraquat, while dopamine levels were quantified using ELISA. Gene expression was analyzed via real-time qPCR. Immunofluorescence staining and neurite length measurements were conducted to validate the PD model and assess cellular morphology.

Results: Cells cultured at a density of $5x10^3$ cells/cm^2 with collagen and 2% FBS exhibited characteristics of dopaminergic neurons upon exposure to retinoic acid. Conversely, paraquat treatment induced neurotoxicity, resulting in decreased dopamine levels and impaired neurite outgrowth.

Conclusion: This study investigated the optimization of SH-SY5Y cell culture conditions for PD modeling. Key findings include optimal cell density, FBS concentration, and beneficial effects of collagen coating. Additionally, an effective paraquat neurotoxicity protocol has been established, providing a solid framework for future research on neuronal differentiation and degeneration.

Key words: Parkinson's disease, SH-SY5Y cells, neuronal differentiation, neurotoxicity, in vitro modeling

ÖZ

Amaç: Bu çalışma, Parkinson hastalığı (PH) araştırmaları için doğru in vitro hastalık modelleri geliştirmek amacıyla SH-SY5Y kültür koşullarını optimize etmeyi amaçlamaktadır. Kollajen kaplama, fetal sığır serum (FSS) konsantrasyonu, diferansiyasyon ajanları ve nörotoksin tedavileri gibi çeşitli faktörlerin hücresel davranış ve hastalık modellemesi üzerindeki etkilerini araştırmayı hedeflemektedir.

Yöntem: İnsan nöroblastoma SH-SY5Y hücre hattı, ısı ile inaktive edilmiş FSS, penisilin-streptomisin ve L-glutamin ile desteklenmiş DMEM/F12 içinde kültürlenmiştir. Hücre diferansiyasyonu üzerindeki etkisini değerlendirmek için kollajen kaplama uygulanmış, ideal hücre yoğunluğu ve serum oranı ise deneysel olarak belirlenmiştir. Parakuat'ın sitotoksik etkilerini değerlendirmek için MTT testi kullanılmış, dopamin seviyeleri ELISA ile ölçülmüştür. Gen ekspresyonu gerçek zamanlı qPCR ile analiz edilmiştir. Parkinson modelini doğrulamak ve hücresel morfolojiyi değerlendirmek için immünofloresan boyama ve nörit uzunluğu ölçümleri yapılmıştır.

Bulgular: $5x10^3$ hücre/cm^2 yoğunluğunda kültürlenmiş hücreler, kollajen ve %2 FSS ile retinoik asit maruziyetinde dopaminerjik nöron özellikleri sergilemiştir. Bununla birlikte, parakuat tedavisi nörotoksisiteye neden olmuş, dopamin seviyelerinde azalma ve nörit büyümesinde bozulma gözlenmiştir.

Sonuç: Bu çalışma, PH modellemesi için SH-SY5Y hücre kültürü koşullarınının optimizasyonu araştırmıştır. Temel bulgular arasında optimal hücre yoğunluğu, FSS konsantrasyonu ve kolajen kaplamanın faydalı etkileri yer almaktadır. Ek olarak, nöronal farklılaşma ve dejenerasyon konusunda gelecekteki araştırmalar için sağlam bir çerçeve sağlayan etkili bir parakuat nörotoksisite protokolü oluşturulmuştur.

Anahtar kelimeler: Parkinson hastalığı, SH-SY5Y hücreleri, nöronal diferansiyasyon, nörotoksisite, in vitro modelleme

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Introduction

The SH-SY5Y cell line, derived from the SK-N-SH cell line isolated from a 4-year-old neuroblastoma patient's bone marrow [1], exhibits a range of human-specific proteins and protein isoforms absent in rodent primary cultures. Known for producing neuroblastic (N-type) cells capable of substrate adhesion (S-type) and transdifferentiation [2], SH-SY5Y cells contain a small fraction of S-type cells despite originating from three consecutive subclones of N-type cells. Several differentiation protocols have been developed to enhance SH-SY5Y as a neuronal cell culture model [3]. Differentiation induces cells into the G0 and G1 phases, leading to synchronized cell cycles and the formation of a homogeneous neuronal population [2], accompanied by specific events such as neuritic structure formation and synaptophysin-positive synapse development [4].

Retinoic acid (RA) addition to the culture medium is a well-established method for SH-SY5Y differentiation, known for its growth inhibitory and differentiation-inducing properties [5]. Typically, RA is applied at 10 µM for 3-5 days in serum-free or low-serum media [4], although protocol variations exist.

Simultaneous stimulation of adhesion and growth factor receptors is emphasized for SH-SY5Y differentiation [6]. The influence of the extracellular matrix (ECM) on neuroblastoma differentiation is evident, with ECM composition changes inducing morphological differentiation [7]. This underscores the role of ECM-derived signals in guiding neuroblastoma differentiation, particularly given the impact of ECM stiffness and biophysical properties on neuroblastoma processes [8]. Moreover, the overlap between signaling pathways involved in the biophysical cross-relationship and RA-mediated neuritogenesis suggests a potential influence of mechano-transductive signals from the ECM on neuroblastoma differentiation [9].

Collagen, fibronectin, and laminin, ECM components, are widely employed in cell culture to enhance cell adhesion. Collagen, in particular, affects cortical progenitor cell proliferation and differentiation, promoting neuronal fate while inhibiting early progenitor cell proliferation [10].

Establishing a reliable human neuron culture method is vital for accurately modeling the human nervous system and studying neurodegenerative diseases. This study aims to determine the optimal RA/collagen/low-serum combination to stimulate adhesion and growth factor receptors simultaneously in SH-SY5Y cells and to establish an in vitro model of PD using paraquat, a neurotoxin.

Materials and Methods

Cell culture

The human neuroblastoma SH-SY5Y cell line (ATCC, Rockville, MD, USA) was acquired from the SAP Institute (Ankara, Turkey). Cells were cultured in a 1:1 mixture of DMEM/F12 supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1% (v/v) L-glutamine and maintained in a humidified CO_2 (5%) incubator at 37°C. Upon seeding, cells were allowed to proliferate for 24 hours until reaching 80–90% confluency, with medium renewal every other day.

Investigation of the effect of collagen during differentiation of SH-SY5Y cells

Some of the six-well plates were coated with 5 µg/cm2 collagen (A10483-01, Gibco) following the manufacturer's instructions, while others were left uncoated. Subsequently, cells were seeded into the wells at a density of $5x10^4$ cells/cm². After a 24-hours incubation at 37°C with 5% CO_2, cells in the wells were treated with RA (10 µM) (R2625, Sigma-Aldrich) supplemented medium containing 2% FBS. The medium was refreshed every other day. Cell images were captured under an inverted microscope (Zeiss Axio) on days 0, 3, and 6.

Determination of optimum cell density and serum ratio in the generation of neurite-like differentiated-SH-SY5Y (d-SH-SY5Y) cells

Six-well plates were coated with collagen, and cells were seeded into the wells at densities of $5x10^3$, $1x10^4$, and $5x10^4$ cells/cm². Following a 24-hours incubation at 37°C with 5% CO^2, cells were treated with medium containing varying proportions of FBS (1% and 2%) and RA (10 µM). The medium was refreshed every other day. On day 6, cell images were captured under an

inverted microscope (Zeiss Axio).

Determination of concentration and time-dependent effects of paraquat on d-SH-SY5Y cell viability by MTT assay

SH-SY5Y cells (passages 20 to 45) were subcultured into 96-well plates at a density of 5x10³ cells/cm². Cells were treated with medium supplemented with 10 μM RA for 6 days to enhance the dopaminergic phenotype and induce differentiation into neurite-like cells. d-SH-SY5Y cells were exposed to different concentrations of paraquat (0, 250, 500, 1000, and 2000 μM) for 24 hours. Subsequently, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) (SE2039502-Serva) solution was added to the wells at a final concentration of 5 mg/ml, followed by further incubation for 4 hours at 37°C, 5% CO². The supernatants were removed, and the pellets were dissolved in 200 μl/well of DMSO. The solubilized blue crystals were measured colorimetrically at 570 nm and 690 nm using a microplate reader (Bio-tek Instruments, Inc.). Cell viability percentage was calculated relative to the colorimetric intensity of control cells.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

The Human Dopamine (DA) ELISA Kit (MBS045009; MyBioSource, San Diego, CA), with a sensitivity of 1.0 pg/mL, was employed to quantify the concentrations of DA in (d)-SH-SY5Y cells following treatment with RA and RA+Paraquat, adhering strictly to the guidelines provided by the manufacturer. Absorbance of samples was recorded at 450 nm. The experimental procedure was conducted in triplicate for validation purposes.

Neurite length measurements

Images were analyzed using ImageJ software, and neurites were manually traced using the ImageJ Plug-In NeuronJ. The cumulative neurite length from all cells and the cell count from the DAPI channel were utilized to calculate the relative neurite length. Additionally, the numbers of NeuN+ cells were determined from images of (d)-SH-SY5Y cells. Three independent cell culture experiments were conducted, and at least three different images were captured for each experiment. At least 50 neurites per condition were quantified for length from the images.

Real-Time Quantitative Polymerase Chain Reaction

Real-time qPCR analyses were conducted following previously established protocol (Bucolo et al., 2012), with slight modifications. Each reaction for the gene of interest was performed in a final volume of 10 μL, comprising 3 μL of cDNA, 0.4 μL of Milli-Q water, 5 μL of SensiFAST SYBR No-ROX master mix (Bioline, Australia), and 0.8 μL of corresponding forward and reverse primers (5 μM, Sigma-Aldrich, Castle Hill, NSW, Australia) to achieve a final primer concentration of 400 nM. The sequences of the primers for the RT-PCR were as follows: NeuN Forward primer (5′-3′): CTACAGCGACAGTTACGGCA, Reverse primer (5′-3′) ATGGTCCGAGAAGGAAACGG; GAPDH Forward primer (5′-3′) CAGCCTCAAGATCATCAGCA, Reverse primer (5′-3′) TGTGGTCATGAGTCCTTCCA.

Immunofluorescence staining of (d)-SH-SY5Y cells with Anti-Tyrosine Hydroxylase antibody

To validate the establishment of an in vitro Parkinson's model, SH-SY5Y cells were seeded in collagen-coated 6-well plates at a density of 5x10³ cells/cm², differentiated with RA treatment for 6 days, and then treated with paraquat (1000 μM) for 24 hours. Following fixation with paraformaldehyde for 15 minutes, cells were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (v/v) and 5% bovine serum albumin for 30 minutes at room temperature. (d)-SH-SY5Y cells were immunostained with NeuN antibody (GeneTex (GTX133127) at a dilution of 1:1000, which labels dopaminergic/noradrenergic neurons. After overnight incubation at 4°C, cells were incubated with secondary antibodies (Alexa Fluor 594 anti-rabbit IgG, Cell Signaling Technologies (8889) at a dilution of 1:250, along with DAPI at room temperature. Cells were observed under a fluorescent microscope at 40X magnification. In order to provide a semi-quantitative analysis of staining intensities, we utilized ImageJ v1.51 (n = 10 images per experimental condition were analyzed for NeuN-IR).

Statistics

Data were derived from a minimum of three independent experiments. Mean values are presented, with error bars indicating the standard error of the mean (SEM). Statistical comparisons between the control group and the treated groups were performed using an unpaired two-tailed Student's t-test for parametric data, or the Mann-Whitney U test for non-parametric data. For multiple group comparisons, one-way ANOVA was utilized, followed by Tukey's HSD post hoc test for parametric data with equal variance, or the Games-Howell post hoc test for parametric data with unequal variance, to conduct pairwise comparisons between the groups. Statistical analysis was performed using the IBM SPSS version 21.0 software package. Statistical significance was set at $p < 0.05$ as appropriate.

Results

Effect of collagen, cell density and serum ratio in forming neuron-like SH-SY5Y cells

The impact of collagen on the differentiation of SH-SY5Y cells was investigated. Enhanced adhesion of (d)-SH-SY5Y cells was observed in collagen-coated wells compared to uncoated wells, resulting in improved cell differentiation. However, at high cell densities, cells were found to be overcrowded, resulting in reduced cell viability by the 6th day (Fig 1A). Therefore, future SH-SY5Y differentiation protocols should be conducted in collagen-coated wells. Furthermore, optimization of the fetal bovine serum (FBS) concentration in the medium and cell seeding density per well were prioritized.

$5x10^3$ cells/cm2

Treatment with medium containing 1% FBS: Although there were cells that adhered and formed neurite-like connections, it was observed that most of the cells lost their adhesion and became suspended.

Treatment with medium containing 2% FBS: It was observed that 80-90% of the cells formed neurite-like connections with neighboring cells, and the cell density per well was appropriate.

$1x10^4$ cells/cm2

Treatment with 1% FBS-containing medium: It was determined that there were less adherent cells compared to the $5x10^3$ cells/cm² group, that the adherent cells did not form neurite-like structures, and that the suspended cells were more.

Treatment with medium containing 2% FBS: It was observed that the cells provided neurite outgrowth, but the cell density in the well was too high.

$5x10^4$ cells/cm²

Treatment with medium containing 1% FBS: It was observed that almost all of the cells were suspended.

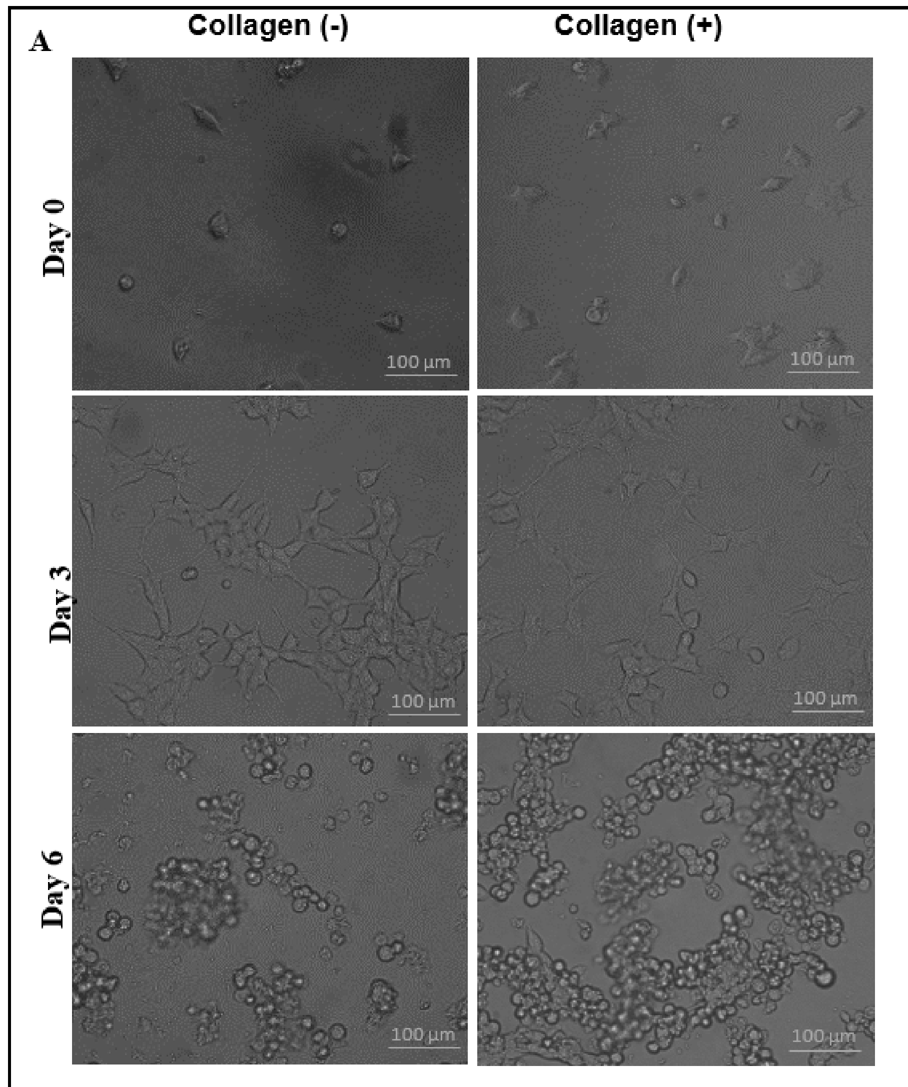
Treatment with medium containing 2% FBS: Compared to the cell group at the same density treated with medium containing 1% FBS, it was determined that the cells had a better tendency to adhere to the ground, but the cell density was higher.

As a result, it was determined that $5x10^3$ cells/cm² density and medium containing 2% FBS were suitable for SH-SY5Y differentiation. It was decided to meet these conditions in future studies (Fig 1B).

Effect of paraquat on (d)-SH-SY5Y cell viability

Paraquat decreased (d)-SH-SY5Y cell viability in direct proportion to concentration and exposure time. There was no statistically significant difference in the viability rates of (d)-SH-SY5Y cells applied 250 or 500 μM paraquat for 24 hours, compared to control (Table 1). However, a statistically significant reduction in cell viability was observed in (d)-SH-SY5Y cells treated with 1000 or 2000 μM paraquat compared to the control (Table 1).

According to ISO 10993-5 standards, four qualitative classification groups were based on to determine the cytotoxic effect levels of chemicals tested in vitro. According to this standard, it is stated that the total cell concentration of highly cytotoxic substances causes cell death above 50%, while 50-79% of substances with moderate cytotoxic effect, 80-89% of substances with mild cytotoxic effect, and 90% of non-cytotoxic substances. It has been stated that it provides cell viability equal to or more than 90% (Iso and STANDARD 2009).



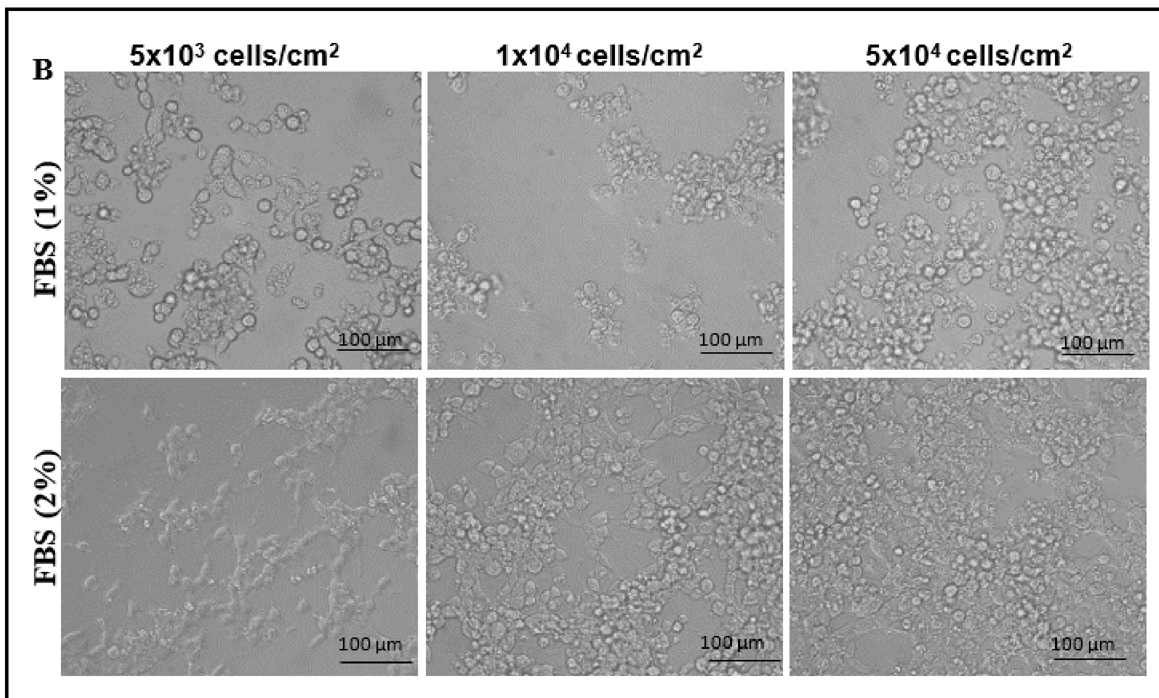


Figure 1: Photomicrographs of SH-SY5Y cells treated with RA in collagen-coated and uncoated wells on days 0, 3, and 6 (A), cultured with different cell density and FBS ratios (B) (20X magnification) (Scale bar: 100 µm).

The 24-hour application of 1000 µM paraquat concentration was determined to be the application that significantly reduced cell viability compared to the control, but did not show a high toxic effect on the cells. Further studies were continued with this application.

Table 1. MTT results of paraquat at 24 hours in each dilution compared to control group

	24 hours		
Groups	Mean of Cell Viability (%)	SD	p value
Control	100	4	-
250 µM Paraquat	98	5	0.950
500 µM Paraquat	95	4	0.445
1000 µM Paraquat	69	2	0.001*
2000 µM Paraquat	50	3	0.001*

SD: standart deviation, *p values compared to control group.

Changes of DA, NeuN and Neurite lengths in (d)-SH-SY5Y cells in response to RA and Paraquat exposure

As presented in Table 2, there was a significant increase in DA concentration in the RA+ group, indicating that RA treatment supports the function of dopaminergic neurons. Conversely, application of paraquat to (d)-SH-SY5Y cells has led to a decrease in the elevated dopamine levels.

The presence of RA, a known neurogenic soluble factor, facilitated the emergence of well-developed neurites. The mean neurite length in the RA-differentiated group showed a significant increase compared to the control group. Paraquat treatment, on the other hand, caused a significant reduction in mean neurite length compared to the RA group only (Table 2).

The study investigated whether RA and paraquat-induced changes in neurite outgrowth correspond with alterations in neuronal marker expression. qRT-PCR analysis demonstrated that the level of NeuN, a neuronal marker, expression in cells treated with both RA and Paraquat was lower than that observed in cells treated solely with RA (Table 2). Immunofluorescence analysis of NeuN exhibited variations consistent with those observed in neurite length and NeuN mRNA expression. RA administration led to robust NeuN immunostaining. However, in the (d)-SH-SY5Y group treated with 1000 µM paraquat for 24 hours following 6 days of RA application, a discernible decrease in NeuN expression level was observed compared to the group receiving only RA (Table 2 and Fig 2).

Discussion

The study's findings encompass the optimization of cell seeding density within SH-SY5Y cell culture conditions aimed at PD modeling. Moreover, it elucidates several critical aspects concerning the necessity of differentiation factors, FBS within the culture medium, collagen for substrate adhesion, and neurotoxic agents for inducing degeneration.

PD is a chronic, progressive disorder characterized by both motor and non-motor symptoms, as well as the loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain and the formation of intracellular inclusion bodies known as alpha-synuclein. It is the second most common neurodegenerative disease worldwide after Alzheimer's disease, with an estimated prevalence of approximately 1% among individuals over the age of 55 [11–13].

In the context of PD models, numerous studies have established the importance of optimizing various parameters such as cell density, differentiation protocols, and serum concentrations to enhance neuronal differentiation and model disease pathologies accurately [14–17]. However, inconsistencies in the literature regarding the optimal conditions for cell differentiation and the precise role of extracellular matrix components, like collagen, highlight a gap in understanding [18–21]. Additionally, the variable effects of FBS concentration on cell proliferation and differentiation, as well as the specific impact of neurotoxins like paraquat on dopaminergic neurons, necessitate further investigation. This study addresses these gaps by systematically optimizing the cell density and FBS concentration for SH-SY5Y cell differentiation, elucidating the effects of collagen coating on cell adhesion and differentiation, and establishing a neurotoxin treatment protocol that balances cytotoxicity and relevance to PD pathology. This research not only corroborates existing findings but also provides new insights into the precise conditions required for reproducible and accurate neuronal differentiation and degeneration modeling in vitro, thereby contributing to the resolution of previously

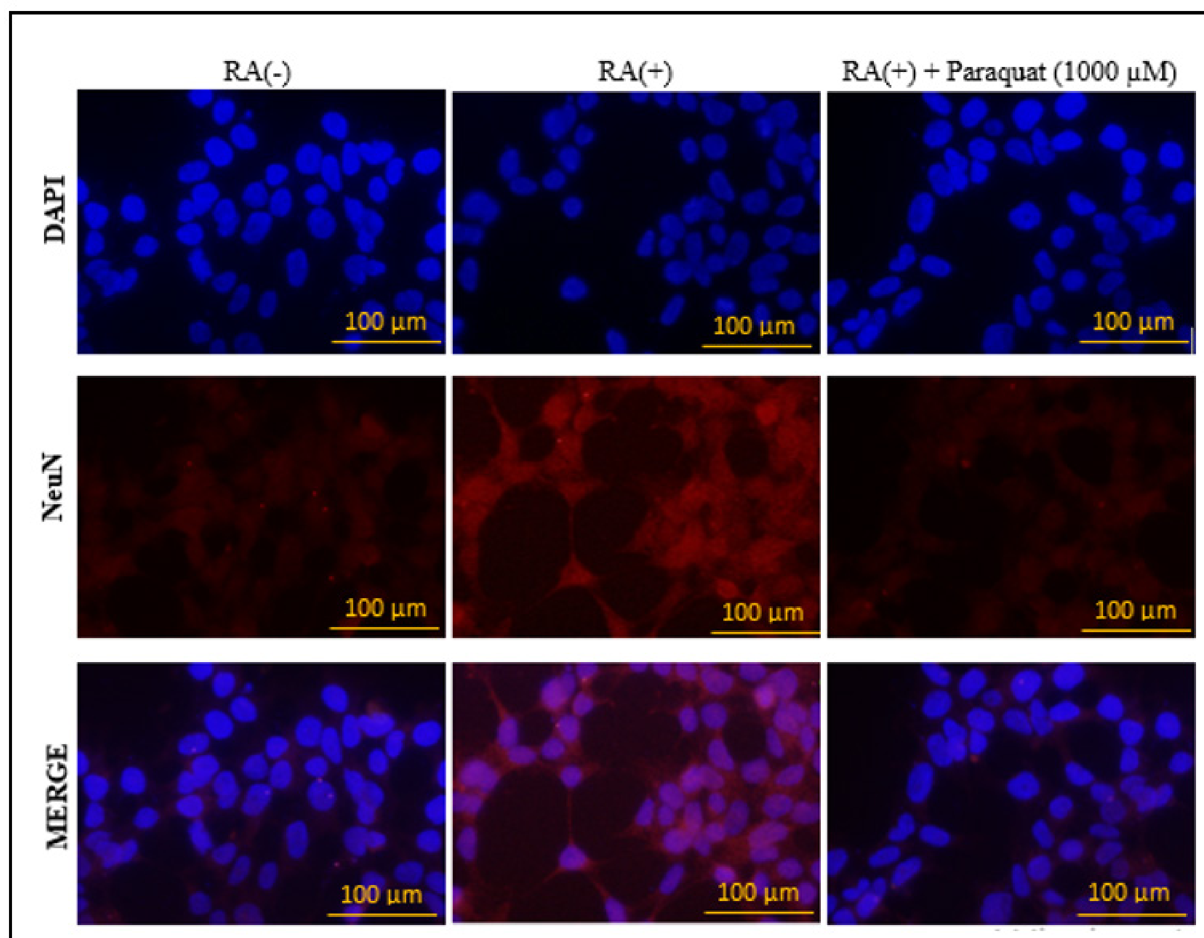


Figure 2: Representative images of NeuN and DAPI immunofluorescence in undifferentiated, retinoic acid differentiated and retinoic acid+paraquat treated SH-SY5Y cells. (40X magnification) (Scale bar: 100 µm).

Table 2. Results on changes in dopamine (DA) concentration, neurite length, mRNA expression level, and integrated density of NeuN in SH-SY5Y cells in response to retinoic acid (RA) and paraquat exposure

	RA (-)			RA (+)			RA (+) + Paraquat		
	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
The concentration of DA (µg/ml)	2	1	-	8	1	0.001*	5	1	0.062#
Mean neurite length (µm)	17	0.5	-	30	0.7	0.001*	20	0.6	0.001#
NeuN mRNA expression (normalized to GAPDH)	1	0.5	-	6	0.8	0.001*	3	0.4	0.002#
NeuN integrated density (arbitrary unit)	10	1	-	29	2	0.001*	10	1	0.001#

Mean: Absorbance (OD), SD: standart deviation, *p values compared to control group, #p values compared to RA (+) group.

conflicting data in the literature.

In this study firstly, the impact of collagen coating on SH-SY5Y cell differentiation was investigated. It was observed that collagen-coated wells promoted enhanced cell adhesion and improved differentiation of SH-SY5Y cells, corroborating previous studies highlighting the importance of extracellular matrix components in neuronal differentiation [22].

FBS, essential for neuronal cell proliferation, differentiation, and maturation, is a vital component of cell culture media, sourced from fetal bovine blood. However, the exact amount of FBS required during the differentiation of SH-SY5Y cells into neurons varies [23]. Deviating from the optimal FBS concentration poses risks; inadequate levels may compromise cell viability and hinder differentiation, while excessive

levels can impede neuronal differentiation by maintaining an undifferentiated state. Additionally, variations in FBS concentration can lead to inconsistent experimental outcomes. Alongside FBS concentration, cell density during neuronal differentiation is critical for orchestrating cellular interactions and signaling pathways essential for functional neuron development [24]. Optimal cell density fosters effective cell-cell communication, promotes neurite outgrowth, and supports synaptic connection establishment, thereby influencing the efficiency and fidelity of neuronal differentiation processes. Deviations from optimal cell density can disrupt these interactions, impairing neuronal maturation and functionality [25]. Hence, meticulous control and optimization of both FBS concentration and cell density are imperative for achieving reproducible outcomes in neuronal differentiation studies conducted in vitro.

Findings to determine the optimal cell density and serum ratio for neurite-like formation in SH-SY5Y cells revealed that a density of 5 x 10^3 cells/cm^2, medium containing 2% FBS, and 6 days of RA application optimally supported neurite growth. These findings are consistent with previous reports indicating the critical role of serum concentration in neuronal differentiation [26] and demonstrating the effect of 2% FBS concentration on neurite elongation in SH-SY5Y cells [27].

The establishment of an optimal neurotoxin treatment protocol, balancing toxicity and relevance to disease pathology, is crucial for the development of a neurodegenerative cell model. The cytotoxic effects of paraquat on (d)-SH-SY5Y cell viability were also assessed in this study. Exposure to paraquat resulted in a decrease in cell viability proportional to concentration and duration; however, it was determined that the most effective neurotoxicity in (d)-SH-SY5Y cells was induced by a 24-hours application of paraquat at a concentration of 1000 μM, which did not exhibit a high degree of toxicity on the cells. These findings are in line with previous studies demonstrating the neurotoxic effects of paraquat [28].

Additionally, changes in DA levels and NeuN expression in response to RA and paraquat exposure were examined. RA treatment led to a significant increase in both DA concentration and NeuN expression. However, exposure to paraquat resulted in attenuation of the RA-induced elevation in dopamine and NeuN levels; this suggests a potential neurotoxic effect on dopaminergic neurons. These findings are consistent with previous studies showing that paraquat causes a decrease in DA level and NeuN expression in neuronal cells [29,30].

Lastly, alterations in neurite lengths further corroborated the neurotoxic effects of paraquat and the neurogenic properties of RA. RA treatment facilitated neurite outgrowth, while paraquat exposure resulted in a significant reduction in neurite length. These findings highlight the potential utility of the SH-SY5Y model established by paraquat administration for studying neuronal development and neurodegenerative processes in PD. In summary, the findings of this study emphasize the complex interaction between environmental toxins and endogenous factors to build a proper and reliable in vitro PD model. Additionally, they offer valuable insights into optimizing SH-SY5Y cell culture conditions for modeling PD.

Limitations

It is important to acknowledge several limitations in this study. Firstly, the use of a single cell line, SH-SY5Y, may limit the generalizability of the findings to other cell types. Future studies incorporating a broader range of cell lines and environmental factors are warranted to address these limitations and enhance the translational relevance of these findings.

Conclusion

In conclusion, this study optimized the culture conditions of SH-SY5Y cells to enhance their utility in PD modeling. We established that a cell density of 5 x 10^3 cells/cm^2, 2% FBS, and six days of RA treatment support optimal neuronal differentiation. Collagen coating significantly enhanced cell adhesion and differentiation. Additionally, a 24-hours exposure to 1000 μM paraquat effectively induced dopaminergic neuron degeneration, aligning with PD pathogenesis. These findings resolve inconsistencies in the literature and establish a robust framework for future research, offering valuable insights for

optimizing in vitro models to study neuronal differentiation and neurodegeneration in PD.

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ORCID and Author contribution: **F.G.K. (0000-0002-7248-7933)** was responsible for formulating the hypothesis, analyses and writing the report.

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