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Defining optimal MTT assay conditions in SH-SY5Y cells: A rotenone-based *in vitro* model of Parkinson's disease

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Although the MTT assay is widely used to evaluate cell viability and cytotoxicity, its results are highly dependent on experimental parameters such as cell density, reagent concentration, and incubation conditions. In neuronal models, particularly in SH-SY5Y cells used for Parkinson's disease studies, a lack of standardized optimization may lead to variability and misinterpretation of results. Therefore, the present study aimed to systematically optimize key parameters of the MTT assay and evaluate their impact under both normal and neurotoxic conditions. To optimize the MTT assay in SH-SY5Y cells, we evaluated the effects of cell number, MTT concentration, incubation time, post-DMSO incubation period, and optical density at different wavelengths. These parameters were further examined in a rotenone-induced *in vitro* Parkinson's disease model. In addition, results obtained from DMSO-treated SH-SY5Y cells were compared under varying assay conditions. Our results indicated that while the MTT method is affected by many parameters, the cell number, MTT concentration, measurement wavelength, and the waiting time after dissolving formazan crystals with DMSO have significant effects on OD values. Consequently, we determined that optimizing this method, which determines cell viability, toxicity, or cell metabolism, is crucial for obtaining and interpreting rational results.

Keywords: Neuroblastoma cells, assay optimization, metabolic activity, dopaminergic neuron model

Introduction

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) is a colorimetric method used to measure cellular metabolic activities[1]. It is also frequently used to measure cytotoxic or cytostatic activities[2]. MTT, which appears yellow when dissolved in water, is converted to insoluble purple formazan by the mitochondrial reductase enzyme[3]. MTT reduction depends on intact electron transport processes within the mitochondrial inner membrane, where NADH-dependent oxidoreductases are key contributors[4]. While the MTT method is frequently used to determine cell viability, it has several limitations[5]. MTT is cytotoxic and can be problematic, particularly in cell viability studies. It is light-sensitive, so working in the dark is important[6]. Formazan crystals treated with solubilizing agents such as DMSO, or isopropanol can lose their stability after a few hours[7].

MTT is one of the most frequently used methods in preclinical studies for the development of anticancer or antineoplastic drugs[8]. Tetrazolium salts are reduced by the oxidoreductase or dehydrogenase enzymes of living cells, converting them into purple formazan (Fig. 1). The amount of formazan formation is directly proportional to the number of living cells[9].

The original MTT method was reported by Mossman in 1983 in which isopropanol was used to dissolve formazan crystals[10]. In several studies different solvents as dimethylformamide, sodium dodecyl sulfate (SDS) and dimethyl sulfoxide (DMSO) were used however it was reported that DMSO is the most powerful solvent to have reliable results[11]. The MTT method consists of several steps. First, a 5mg/mL MTT solution is prepared in PBS. It is then filtered and sterilized through 0.2µm filters. After the desired application is applied to the cells seeded in the 96-well plate 0.5mg/mL of reagent is added to the wells[12]. After incubation for 1-4 hours, the supernatant is withdrawn and 100µl of DMSO is added. Measurements are determined at 570 nm using an ELISA reader. Three replicates of each application are essential for the reliability of results[6]. Obtaining stable OD values is crucial for determining cell viability or cytotoxic effects. Cell viability is calculated by multiplying the ratio of the OD values of control cells to the OD values of treated cells by

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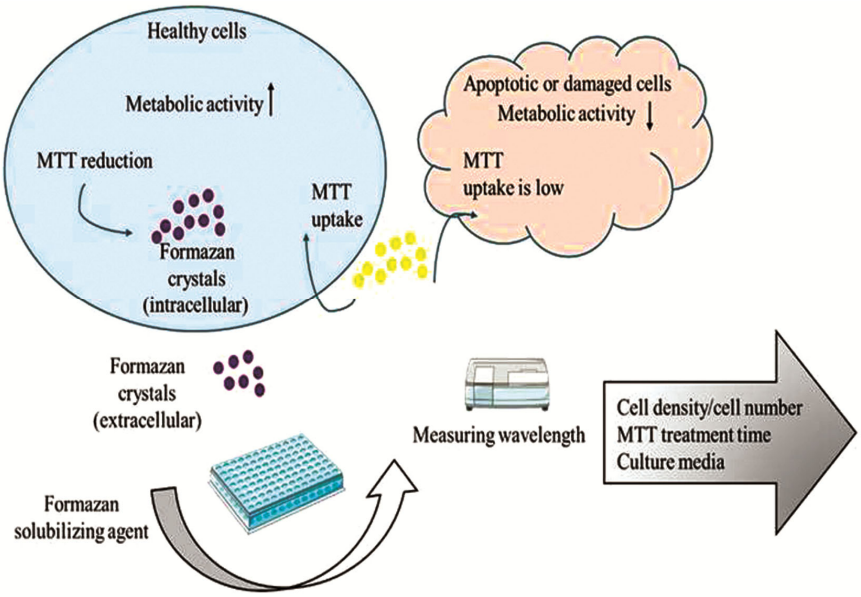

Fig. 1 — Schematic representation of the MTT assay mechanism. In healthy and metabolically active cells, yellow MTT tetrazolium salt is efficiently taken up and reduced by mitochondrial dehydrogenases to insoluble purple formazan crystals, which accumulate intracellularly. Conversely, apoptotic or damaged cells exhibit reduced metabolic activity, resulting in lower MTT uptake and limited formazan formation. The generated formazan is subsequently solubilized and quantified spectrophotometrically, with absorbance values correlating with cell viability and proliferation. Key experimental parameters influencing the assay outcome include cell density/number, MTT treatment duration, and the type of culture medium used.

100[13]. The absorbance spectra of MTT could be changed between 500 nm to 700 nm and around 560 nm was found most reliable[14].

Parkinson is the second most common neurodegenerative disease after Alzheimer's. Its etiology consists of oxidative stress, neuro-inflammation, toxic factors, and genomic factors[15]. Rotenone is a pesticide and an inhibitor of mitochondrial complex I. Among experimental Parkinson's models, the rotenone model provides the closest neuropathological features. Rotenone is used to create a Parkinson's model in rats, and *in vitro*, it has been applied to SH-SY5Y cells, forming the basis of various preclinical studies[16,17].

Considering these advantages and limitations, the MTT assay remains a widely used, cost-effective, and practical method in cell-based studies; however, proper optimization is essential to ensure reliable results. Therefore, the aim of the present study was to systematically optimize key parameters of the MTT assay in SH-SY5Y cells. To achieve this, a rotenone-induced *in vitro* Parkinson's disease model was employed, and cell viability was evaluated under varying experimental conditions. The findings are intended to provide a standardized framework for improving the accuracy and reproducibility of MTT-based measurements in neuronal models.

Materials and Methods

Determination of optimum cell number, MTT concentration, Wavelength, MTT and DMSO treatment time on SH-SY5Y Cells

SH-SY5Y cells grew in 10% FBS, 1% Penicillin-Streptomycin, 1% L-glutamine included DMEM medium. The cells were counted by Trypan blue method and 3000, 5000, 7000 and 10000 cell per well were inoculated to 96 well plates. MTT reagent was prepared in PBS as 5mg/mL main stock and then 0.1, 0.2, 0.3, 0.4, 0.5 mg/mL solutions were prepared. This study was conducted using cell culture techniques only.

Determining the effect of DMSO percentages on SH-SY5Y cells

After determining 570 nm as the optimal wavelength, subsequent DMSO experiments were conducted at this wavelength. DMSO solutions at concentrations of 100%, 10%, 1%, 0.1%, and 0.01% were prepared and applied to SH-SY5Y cells seeded at densities of 3,000, 5,000, 7,000, and 10,000 cells per well. Following 24 hours of exposure to the different DMSO concentrations, 0.5 µg/mL MTT was added to each well, and the plates were incubated for either 2 or 4 hours prior to measurement. To optimize the MTT concentration, SH-SY5Y cells were seeded at a density of 5,000 cells per well in 96-well plates.

After 24 hours of incubation, the culture medium was removed, and DMSO was added to each well. Absorbance was measured at 570 nm following a 15-minute incubation to evaluate formazan formation.

Determining the effect of Rotenone on SH-SY5Y cells

Different wavelengths, MTT concentrations, rotenone concentrations, and incubation times (5, 10, 15, and 30 minutes following DMSO addition) were evaluated. Rotenone was dissolved in DMSO and serial dilutions were prepared at final concentrations of 20, 10, 5, 2.5, and 1.25 µg/mL. SH-SY5Y cells were seeded into 96-well plates at a density of 5,000 cells per well and incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cell attachment. Subsequently, cells were treated with the designated concentrations of rotenone for 24 hours. Following treatment, MTT reagent was applied at final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5µg/mL. The plates were then incubated for either 2 or 4 hours, after which formazan formation was solubilized with DMSO. Absorbance values were measured at 540, 570, and 590 nm under different incubation conditions.

Determining the effect of FBS concentration on rotenone and DMSO treatment

SH-SY5Y cells were seeded into 96-well plates at a density of 5,000 cells/well and allowed to attach for 24 h under standard culture conditions. To evaluate the influence of serum concentration on rotenone-induced cytotoxicity and MTT assay performance, cells were cultured in media containing 10%, 1%, or 0% fetal bovine serum (FBS). Following serum adjustment, cells were treated with rotenone at final concentrations of 20, 10, 5, 2.5, and 1.25 µM for 24 h. After treatment, the culture medium was removed, and MTT solution was added at a final concentration of 0.5 mg/mL. Cells were incubated with MTT for 4 h at 37°C. Subsequently, the supernatant was discarded and the resulting formazan crystals were dissolved in 100 µL DMSO. Absorbance values were measured at 570 nm after 15 min of incubation at room temperature. Cell viability was calculated relative to untreated control cells cultured under the corresponding serum conditions. All experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (ANOVA) was used to evaluate differences between groups. Data are presented as mean ± standard deviation (SD), and $P<0.05$ was considered statistically significant.

Results

Influence of Cell Density, Wavelength, and Incubation Parameters on MTT Assay Optimization

Extending the MTT incubation time significantly affected formazan formation, which depends on cell viability. OD values measured after two hours of incubation increased depending on cell number and MTT concentration (Fig 2). However, absorbance values obtained under the same conditions at four hours of incubation were higher, and differences between cell number and MTT concentration became more pronounced (Fig 3). Especially at high cell densities (7000 and 10000 cells/well) and high MTT concentrations (0.4–0.5mg/mL), OD values measured after four hours of incubation yielded stronger signals compared to those after two hours. This suggests that formazan accumulation due to mitochondrial activity increases with prolonged incubation. When evaluated in terms of the post-DMSO incubation period, OD values increased at 15 and 30 min for both incubation periods, and the separation between concentrations was more clearly observed. However, this effect became more pronounced at the 4h incubation, and differences between concentrations were more consistently separated, particularly at wavelengths 570–590 nm. When wavelengths were compared, OD values measured at 570 nm and 590 nm for both 2h and 4h incubations showed higher signal intensity and lower variation compared to those at 540 nm. This finding supports the more reliable detection of dissolved formazan in the 570–590 nm range. Overall, the findings suggest that extending the MTT incubation time (4h) makes differences in both cell number and MTT concentration more pronounced and increases measurement sensitivity. Therefore, 4-hour MTT incubation, 15–30 minutes of measurement time after DMSO, and use of 570–590 nm wavelengths were determined as the most appropriate conditions for cell viability measurements.

Two-way ANOVA results of (Fig 3) demonstrated that cell number was the main determinant of OD variation, accounting for 90.0% and 93.1% of the total variance at 2 h and 4h incubation, respectively ($P < 0.0001$) (Table 1). MTT concentration also showed a significant effect, explaining 5.2% (2 h) and 4.9% (4h) of the variation ($P< 0.0001$). Moreover, the

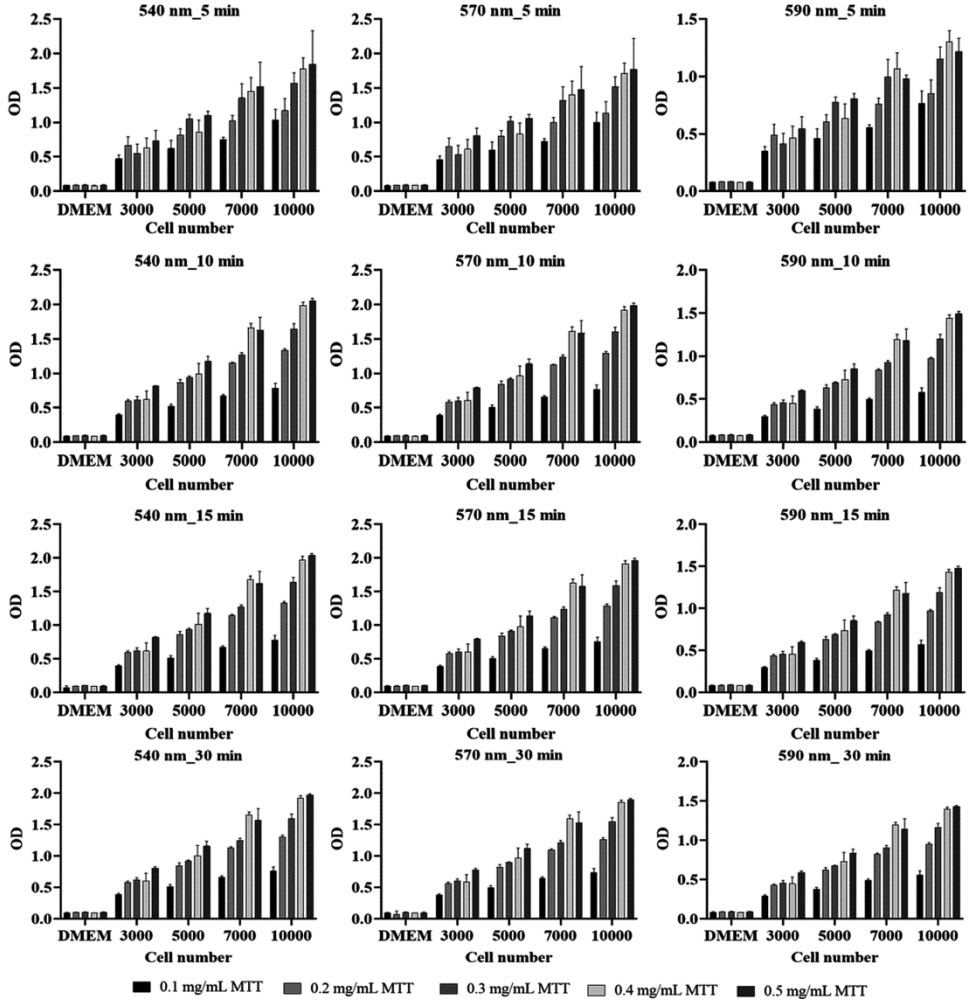

Fig. 2 — Optical density (OD) depends on cell seeding density, MTT concentration, and incubation time. OD values were measured after 2h of MTT incubation using different cell numbers (3,000–10,000 cells/well) and MTT concentrations (0.1–0.5 mg/mL). Measurements were performed at three different wavelengths (540, 570, and 590 nm) at 5, 10, 15, and 30 minutes following DMSO addition. The graphs illustrate changes in OD values with increasing cell density and MTT concentration. Data are presented as mean ± standard deviation (n = 3).

interaction between cell number and MTT concentration was statistically significant at both time points (2h: P=0.0002; 4h: P<0.0001), indicating that the effect of MTT concentration on OD values depended on the cell density. Collectively, these results confirm that both cell seeding density and MTT incubation time significantly influence assay outcomes, with cell number being the predominant factor.

OD values measured in SH-SY5Y cells seeded at different numbers (3000–10,000 cells/well) increased in parallel with the increase in cell number. Furthermore, extending the MTT incubation time (2 h - 4h) resulted in a significant increase in OD values (Fig 4). When comparing wavelengths, 570 nm measurements showed higher sensitivity than other wavelengths. These findings suggest that both cell number and MTT incubation time significantly affect the signal obtained from the MTT assay.

Effect of MTT concentration, cell number and incubation time after MTT treatment on cell death triggered by DMSO

We determined the influence of DMSO concentration, cell number, MTT incubation time and MTT concentration on the outcome of the MTT assay. It was known that DMSO reduces cell viability by more than 1% concentration. OD values increase proportionally with cell number, confirming the sensitivity of the assay to the cell density. Extending incubation times after MTT treatment significantly enhances the signal and provides clear discrimination

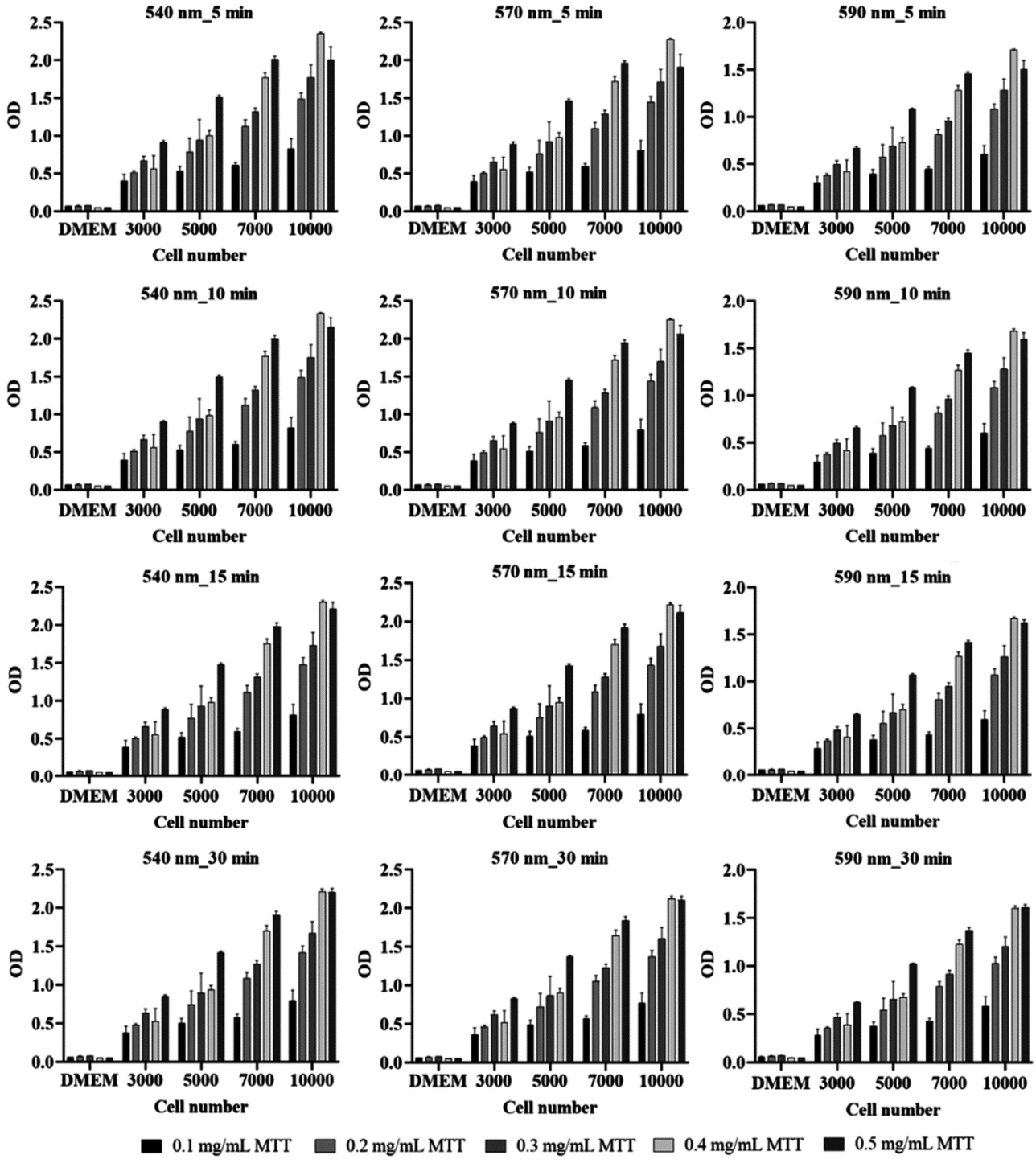

Fig. 3 — Optical density (OD) depends on cell seeding density, MTT concentration, and incubation conditions (4h). OD values were measured after 4h of MTT incubation using different cell numbers (3,000–10,000 cells/well) and MTT concentrations (0.1–0.5 mg/mL). Measurements were performed at three different wavelengths (540, 570, and 590 nm) at 5, 10, 15, and 30 minutes following DMSO addition. The graphs illustrate changes in OD values with increasing cell density and MTT concentration. Data are presented as mean ± standard deviation (n = 3).

Table 1 — Two-way ANOVA results for different cell numbers and MTT concentrations after 15 min of DMSO addition

Source of Variation	% of total variation		F (DFn, DFd)		*P* value		Significance	
	2h	4h	2h	4h	2h	4h	2h	4h
Interaction Cell numberx MTT	1.847	1.784	F (8, 29) = 5.809	F (8, 30) = 30.53	0.0002	*P*<0.0001	***	****
Row Factor Cell number	90.02	93.13	F (4, 29) = 566.3	F (4, 30) = 3187	<0.0001	*P*<0.0001	****	****
Column Factor MTT	5.196	4.870	F (2, 29) = 65.37	F (2, 30) = 333.3	<0.0001	*P*<0.0001	****	****

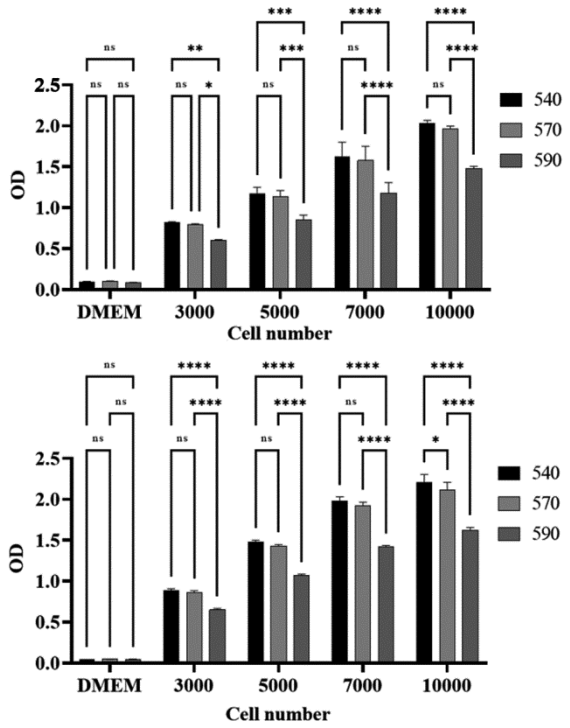

Fig. 4 — Effect of cell density, MTT incubation time, and wavelength on OD measurements in SH-SY5Y cells. Cells were seeded at different densities (3,000–10,000 cells/well) and subjected to MTT assay with 0.5 mg/mL MTT. OD values were measured at 540, 570, and 590 nm after 2 h (left) and 4 h (right) of incubation. OD values increased proportionally with cell density, and prolonged MTT incubation (4 h) further enhanced the signal. Among the wavelengths tested, 570 nm yielded the highest sensitivity. Data are shown as mean ± SD (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (compared to control group); ns, not significant.

between experimental conditions. Together these results highlight that longer MTT incubation improves the dynamic range of the MTT assay (Fig 5).

Evaluating the effect of MTT concentration, wavelength, DMSO incubation times in rotenone treated SH-SY5Y cells

OD levels in cells treated with rotenone between 1.25 and 20μM were compared, different MTT concentration, different wavelength and different incubation times after adding DMSO were determined. 5000cell/well was plated into 96 well plates (Fig 6). Data demonstrate that while OD values increased with MTT concentration, 15–30 min post-

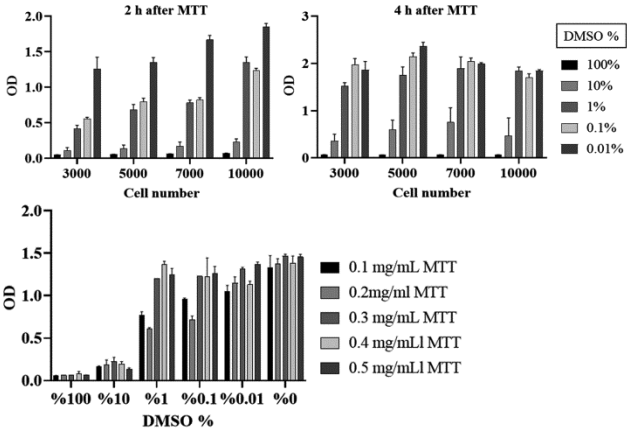

Fig. 5 — *DMSO treated cells' OD levels were determined after 2h and 4 h later than MTT treatment. The results were obtained by adding 0.5μg/mL MTT solution. After DMSO addition 15 minutes incubation was performed and the data analyzed at 570 nm. **DMSO treated cells' OD levels were determined after 4h later than different concentrations of MTT reactive. After DMSO addition 15 minutes incubation was performed and data analyzed at 570nm.

DMSO incubation yielded more stable and consistent absorbance readings. Among wavelengths, 570 nm and 590 nm provided higher sensitivity and reproducibility compared to 540 nm. Values represent mean ± SD of triplicates.

5000cells/well were plated and Rotenone with different concentrations were treated to cells. MTT incubation times (2 and 4) were measured at 570 nm. As shown in (Fig 7), extending the MTT incubation time from 2 hours to 4 hours further enhanced OD values, suggesting that prolonged incubation allows more complete reduction of MTT by metabolically active cells. Error bars represent standard deviations (n = 3), confirming the reproducibility of the results.

Effect of FBS percentages on rotenone induced SH-SY5Y cells were determined by growing the cells up at 10%, 1% and 0% FBS. It was obviously detected that there were no statistically significant differences in FBS percentages and OD levels (Fig 8).

Discussion

In this study, the MTT assay was used to assess cell viability. It should be noted that the assay relies

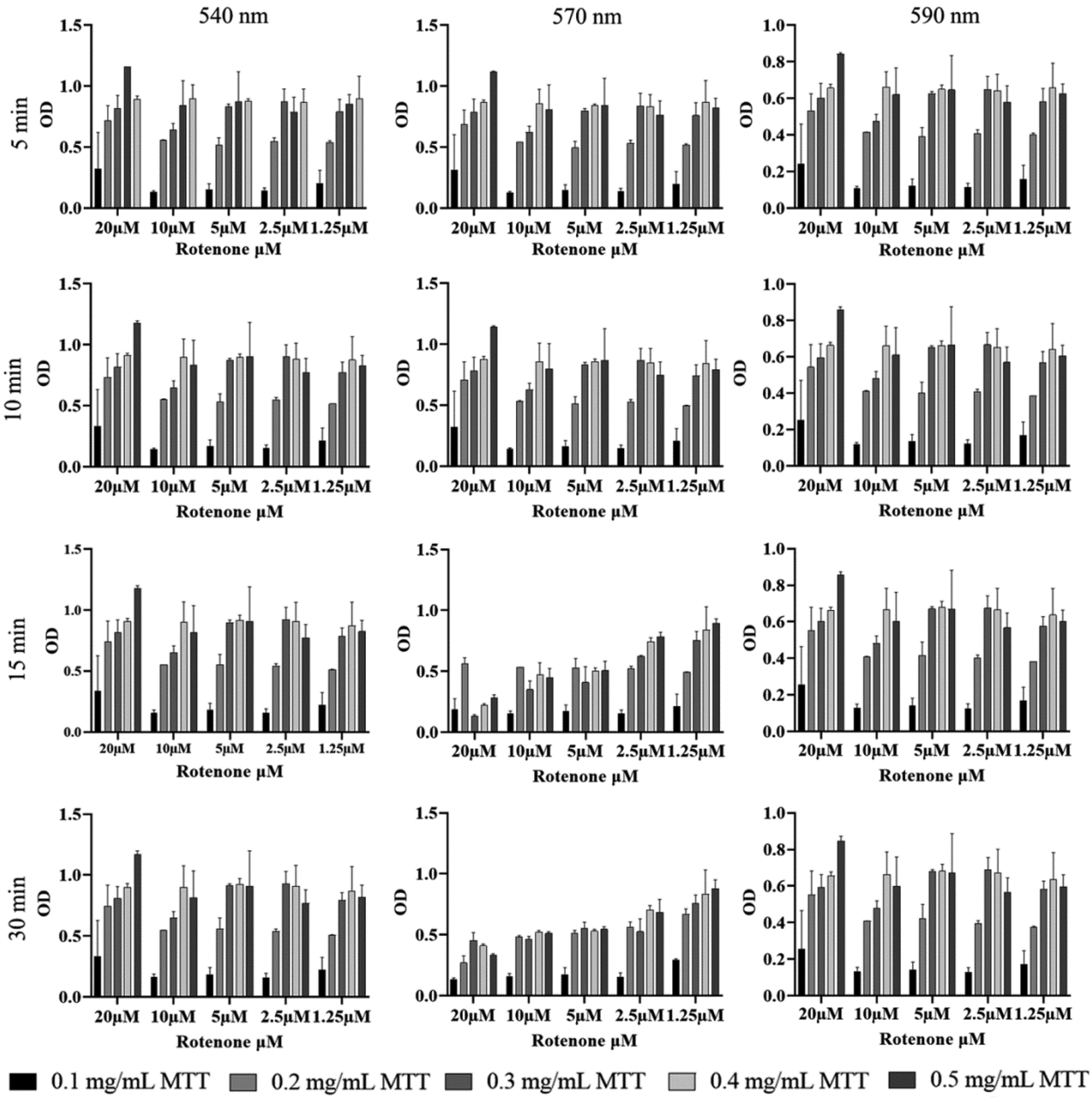

Fig. 6 — Optimization of MTT assay conditions at different wavelengths and post-DMSO incubation times after rotenone treatment. Optical density (OD) values were measured at 540 nm, 570 nm, and 590 nm in 5000 seeded cells treated with varying concentrations of MTT (0.1–0.5 mg/mL). Measurements were performed at 5, 10, 15, and 30 minutes after dissolving formazan crystals in DMSO.

on mitochondrial metabolic activity, which is widely accepted as an indirect indicator of viable cells. Accordingly, the results are interpreted in terms of cell viability while reflecting underlying metabolic activity. The MTT assay remains one of the most widely used methods for evaluating cell viability and cytotoxicity due to its dependence on membrane-bound and mitochondrial enzyme activity. Its continued use is largely attributable to the straightforward detection of formazan crystals formed during the reduction process in metabolically active cells. These insoluble crystals may accumulate in mitochondria, the cytoplasm, or the plasma membrane and are subsequently dissolved using appropriate solvents. Belyanskaya et al. compared two MTT protocols using ethanol and isopropanol/HCl as

solvents while assessing the effects of carbon nanotubes on A549 cells. Although both solvents were found to be suitable, the study highlighted that nanotubes purified by different methods were capable of reducing MTT even in the absence of cells[18]. Similarly, it is known that certain agents, including plant extracts or thiol-containing compounds, can reduce MTT independently of cellular activity[12]. For this reason, careful optimization of MTT assay parameters is essential regardless of the material being studied.

Recent studies further support the importance of optimizing MTT assay conditions in neuronal and neuroblastoma-derived cell models. For example, a 2024 study investigating siRNA delivery in SH-SY5Y cells applied specific assay conditions, including defined cell density (5 × 10^4 cells/well), MTT concentration (0.5mg/mL), and incubation time (3h), highlighting that these parameters are tightly controlled to ensure reproducible metabolic readouts[19]. Similarly, multiple recent neurotoxicity and neuroprotection studies in SH-SY5Y cells continue to rely on the MTT assay but employ varying incubation times and treatment durations depending on the experimental design, demonstrating that assay conditions are not universal and must be adapted to each model[20,21]. In addition, recent work on neurotoxin exposure in neuroblastoma cells has shown that even low concentrations of compounds can alter metabolic activity and apparent cell viability in MTT assays, further emphasizing the sensitivity of the method to experimental parameters[22]. Together, these findings indicate that without careful optimization of variables such as cell density, incubation time, and assay conditions, MTT results may vary significantly across studies. Therefore, the optimized parameters established in the present study may provide a useful reference framework for improving consistency and interpretability in other neuronal and neuroblastoma-based experimental systems.

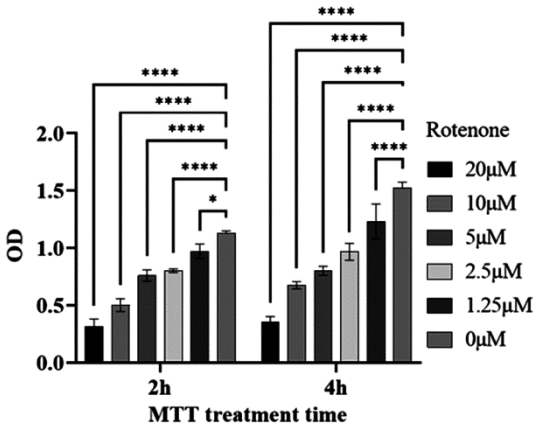

Fig. 7 — Rotenone treated cells OD levels determined after 2h and 4h later than MTT treatment. 5000cell/well, 0.5μg/mL MTT solution was performed. After DMSO addition 15 minutes incubation was performed. Data analyzed at 570 nm. *P < 0.05, **P < 0.01, ***P < 0.001 (compared to control group); ns, not significant.

It has been demonstrated that cultivation conditions significantly influence the growth characteristics and metabolic activity of SH-SY5Y and other neuronal cell models, thereby directly affecting assay outputs. For instance, a 2024 study showed that factors such as serum concentration, extracellular matrix coating, and differentiation protocols can markedly alter cell behavior and experimental outcomes in SH-SY5Y-based Parkinson's disease models[23]. Similarly, recent

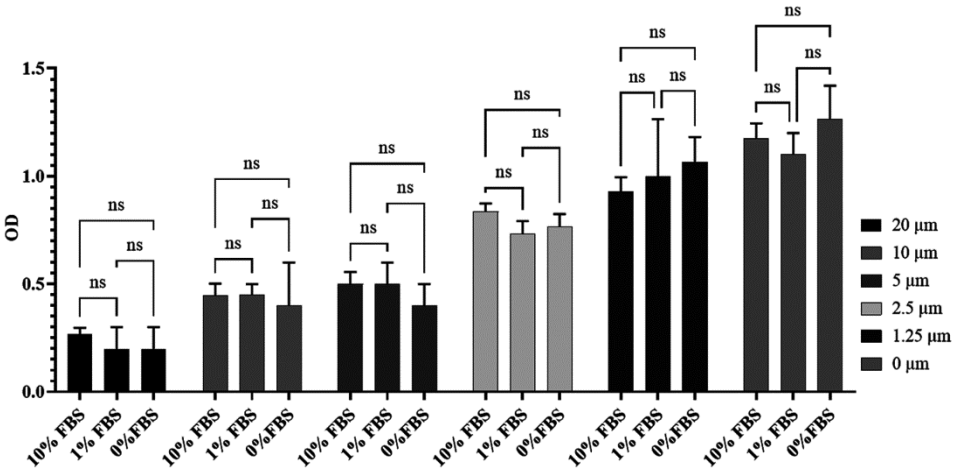

Fig. 8 — FBS percentage effect on rotenone induction. No significant effect of 10%, 1% and 0% FBS have been detected on rotenone induced cells. *P < 0.05, **P < 0.01, ***P < 0.001 (compared to control group); ns, not significant.

experimental studies report that variations in seeding density (e.g., 2×10^4–5×10^4 cells/well) and incubation conditions can substantially impact proliferation and metabolic readouts measured by assays such as MTT [24].In addition, recent analyses emphasize that the lack of standardized experimental parameters across studies contributes to variability and limits reproducibility in neurotoxicity models using SH-SY5Y cells[25].These findings highlight that differences in cultivation conditions directly affect cellular yield and metabolic activity, underscoring the necessity of optimizing assay parameters. Therefore, the optimized conditions established in the present study may contribute to improving consistency and interpretability of MTT-based measurements in neuronal *in vitro* systems.

In the present study, we first identified the parameters affecting SH-SY5Y cell growth and optimal cell density at 24 hours. Variables such as cell number, MTT concentration, incubation time, post-DMSO incubation duration, and optical density (OD) readings at different wavelengths were systematically evaluated. Four main observations emerged from the analysis. First, the choice of reading wavelength has a strong influence on signal intensity and the ability to distinguish between experimental groups. Second, a minimum of 15 minutes after DMSO addition is required for consistent and reliable solubilization. Third, both MTT concentration and cell density significantly affect signal magnitude, although excessively high values may lead to saturation. Finally, optimization of cell number is critical, as it directly influences viability and cytotoxicity outcomes; therefore, appropriate ranges should be established for each cell type. These trends were consistent across all tested wavelengths, although differences in signal strength, variability, and dynamic range helped define optimal assay conditions.

The selection of wavelength is known to influence MTT assay outcomes, with 570 and 590nm commonly used for detecting formazan[13]. Abbasi *et al.* evaluated SiO_2 nanoparticle effects at wavelengths of 470, 490, 520, and 570nm, reporting the most sensitive and reliable results above 550 nm[26]. In line with these findings, our results—both in untreated SH-SY5Y cells and in rotenone-induced models—show that measurements at 570 nm yield the highest and most consistent OD values, along with the clearest separation between experimental groups. While readings at 540 and 590 nm reflected similar overall trends, they generally produced lower signal intensity and greater variability, particularly at earlier time points. Given that the absorption maximum of MTT formazan is typically near 570 nm, the superior performance of this wavelength is expected, as it enhances signal-to-noise ratio and improves discrimination between experimental conditions.

Following DMSO addition, the temporal progression of solubilization indicates that 5 and 10 minutes are often insufficient for complete dissolution of formazan crystals. At these early points, OD values tend to be lower and more variable. By 15 minutes, OD readings increase and variability decreases, indicating more consistent solubilization. Measurements at 30 minutes are comparable to those at 15 minutes, suggesting that the signal has reached a stable plateau. These observations indicate that a minimum solubilization period of 15 minutes is required for reliable measurements. Extending this period to 30 minutes does not significantly alter results but may provide additional assurance of complete dissolution. This pattern is consistent with previous studies, including bacterial MTT assays, where longer dissolution times reduce variability and improve consistency[27].

Our findings also demonstrate a clear relationship between OD values, MTT concentration, and cell density. Increasing either parameter leads to higher OD signals, thereby expanding the assay's dynamic range. However, at higher MTT concentrations and cell densities, the response may become non-linear or approach saturation, particularly when measurements are taken at suboptimal wavelengths or before complete solubilization. The results suggest that using an intermediate MTT concentration, together with an appropriate range of cell numbers within the linear response range and measuring absorbance at 570 nm after at least 15 minutes of DMSO incubation, provides the most reliable and quantitative results.

Overall, the experiments identify key parameters that improve assay sensitivity and reproducibility. Measuring absorbance at 570 nm, allowing at least 15 minutes for formazan solubilization (with 30 minutes as an acceptable alternative), and selecting MTT concentrations and cell densities that remain within the linear range are critical for optimal performance. Measurements performed at 540, 570, and 590 nm at different time points (5, 10, 15, and 30 minutes) consistently showed that 570 nm readings taken after

at least 15 minutes provided the highest dynamic range and lowest variability. Earlier time points and non-optimal wavelengths resulted in weaker and less consistent signals. Based on these findings, subsequent quantitative MTT assays were conducted using 570 nm and a minimum solubilization period of 15 minutes.

These findings highlight that even minor variations in experimental conditions can lead to substantial differences in MTT assay outcomes, which may contribute to inconsistencies across studies. Therefore, standardized optimization protocols are essential not only for improving reproducibility but also for ensuring accurate interpretation of cell viability and metabolic activity in neuronal models. Furthermore, given the widespread use of MTT assays in neurodegenerative disease research, particularly in Parkinson's disease models, the optimized parameters defined in this study may serve as a practical reference for future *in vitro* investigations.

Conclusion

In conclusion, this study systematically optimized key parameters of the MTT assay in SH-SY5Y cells, including cell density, MTT concentration, incubation time, post-DMSO solubilization period, and measurement wavelength. The results demonstrated that absorbance at 570 nm, a minimum of 15 minutes post-DMSO incubation, and the use of appropriate cell density and MTT concentration are critical for obtaining reliable and reproducible measurements. These findings address the need for standardized assay conditions highlighted in the introduction and provide a practical framework for improving the accuracy and consistency of MTT-based viability assessments, particularly in neuronal and Parkinson's disease *in vitro* models.

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