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Research Paper

Biphasic Neuroprotective and Pro-Inflammatory Synergistic Effects of Red Laser Photobiomodulation Combined with Levodopa in a Rotenone-Induced SH-SY5Y Cell Model of Parkinson's Disease: An in Vitro Study

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ABSTRACT

Background: Parkinson's disease is a progressive neurodegenerative disorder with no curative therapy. Red laser photobiomodulation (PBM) has emerged as a potential neuroprotective strategy because of its antioxidant and mitochondrial biostimulatory properties. To evaluate the neuroprotective efficacy of individual and combined levodopa and red laser PBM pre-treatment against rotenone-induced neurotoxicity in SH-SY5Y neuroblastoma cells.

Methods: SH-SY5Y cells were pre-treated with levodopa (25, 50, 100 µM), continuous-wave red laser PBM (630–660 nm, 100 mW, 0.5 W/cm²; 10, 20, 40 s), or their combined configurations for 2 h, followed by a 24-hour rotenone (50 µM) challenge. Cell viability (MTT), IL-1β (ELISA), MDA (TBARS), and total antioxidant capacity (TAOC) were quantified.

Results: Rotenone induced cytotoxicity, elevated IL-1β and MDA levels, and depleted TAOC. Individual Levodopa (50 µM) reduced MDA ($P < 0.0001$) and restored TAOC ($P < 0.0001$) but failed to rescue viability ($P > 0.05$). A 20-second red laser exposure (2 J, 10 J/cm²) optimally enhanced cell survival ($P = 0.0087$), reduced lipid peroxidation ($P = 0.0002$), and recovered TAOC ($P = 0.0004$), demonstrating a biphasic dose-response. Specific combined regimens (Triple Pre 1 and 2) triggered a synergistic surge in IL-1β ($P < 0.0001$), driven by hypermetabolic cross-talk between levodopa auto-oxidation and PBM biostimulation, whereas Triple Pre 3 maintained cytokine homeostasis ($P > 0.05$ vs. rotenone control).

Conclusion: Red laser PBM provides biphasic, dose-dependent neuroprotection by restoring bioenergetics and antioxidant defenses; however, its combination with levodopa can provoke severe pro-inflammatory synergy, necessitating strict dosimetric thresholding in multimodal Parkinson's interventions.

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Introduction

Parkinson's disease (PD) is a chronic, age-related neurodegenerative disorder and is considered the second most common neurodegenerative disease worldwide, affecting millions of individuals [1]. The prevalence of PD accounts for nearly 2% of the aging population, and despite extensive research, no definitive curative therapy is currently available [2–4]. The disease affects both motor and non-motor functions of the central nervous system and is primarily characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta, along with dopamine depletion in the striatum [5]. These pathological alterations lead to the appearance of major clinical manifestations, such as tremors, muscular rigidity, bradykinesia, and impaired movement coordination [6].

Among the experimental models used in neuroscience research, the SH-SY5Y human neuroblastoma cell line is one of the most widely utilized in both undifferentiated and differentiated forms [7]. Furthermore, PD-related cellular damage can be induced in SH-SY5Y cultures either through exposure to neurotoxins such as rotenone (ROT), which produces PD-like pathological alterations, or through genetic modification techniques that promote the overexpression of pathogenic proteins associated with the disease [7]. SH-SY5Y cells possess catecholaminergic properties and express enzymes, including tyrosine hydroxylase and dopamine β-hydroxylase, enabling them to synthesize dopamine and noradrenaline neurotransmitters [8].

Red laser photobiomodulation (PBM) is a noninvasive therapeutic modality that modulates cellular functions by interacting with mitochondrial cytochrome c oxidase. This interaction enhances electron transport, restores the mitochondrial membrane potential, and increases ATP production without causing thermal damage [9, 10]. Consequently, PBM has emerged as a promising strategy for promoting neuronal survival, reducing oxidative stress, and mitigating neuroinflammation in neurodegenerative conditions, including Parkinson's disease [11].

Given that current pharmacological approaches, such as levodopa, provide symptomatic relief but carry long-term limitations, exploring multimodal interventions has become crucial. While individual red laser photobiomodulation has shown promise in enhancing bioenergetics, its synergistic effects with exogenous catecholamines remain poorly characterized. Therefore, this study was designed to systematically evaluate the neuroprotective potential of individual levodopa, red laser PBM, and their novel combined configurations (designated as Triple Pretreatments) against rotenone-induced cytotoxicity, neuroinflammation, and oxidative stress in SH-SY5Y neuroblastoma cells. This approach aims to establish optimized therapeutic thresholds and uncover potential metabolic cross-talk for enhanced neuroprotection in Parkinson's disease models.

Materials and Methods

Cell culture and maintenance

Human neuroblastoma SH-SY5Y Cells were obtained from the College of Medicine, University of Babylon, Iraq. Cells were cultured in RPMI medium (Gibco, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, UK) and 80 mg gentamicin (Arab Pharm, Jordan). Cells were maintained at 37 °C in a humidified incubator under a 95% air and 5% CO_2 atmosphere. Cells were allowed to reach approximately 70% confluence before being used for experimental procedures.

Cell culture supernatants and lysates were collected for biochemical and immunological analyses using ELISA and colorimetric assays, including malondialdehyde (MDA), interleukin-1 beta (IL-1β), and total antioxidant capacity (TAOC).

All experiments were performed in five independent biological replicates (n = 5) per experimental group. The sample size was determined based on previous in vitro PBM studies employing similar neuroblastoma cell models [12,14, 25], which demonstrated that five replicates per group provide adequate statistical power (≥80%) to detect biologically meaningful differences (effect size d ≥ 1.5) at a significance level of α = 0.05 using one-way ANOVA with post-hoc comparisons. Although formal blinding and randomization procedures are not standard practice in routine in vitro cell culture assays of this nature, all measurements were performed using automated plate readers to minimize operator-dependent bias, and treatment allocations were coded during data acquisition and analysis to reduce potential assessment bias.

In Vitro Study Design and Cell Viability Assay (MTT Assay)

Cell survival, cytotoxicity baseline, and protective efficacy of the pre-treatment protocols were comprehensively evaluated using the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay according to standard procedures.

Optimization of Rotenone Cytotoxicity

To establish an in vitro Parkinson's disease model, a preliminary pilot study was conducted in which SH-SY5Y cells were challenged with serial dilutions of rotenone ranging from 0.62 to 80 µg/mL for 24 h. Cell viability was measured to determine the optimal cytotoxic concentration (50 µM) required to induce steady and reproducible neurotoxicity [12].

Optimization of Levodopa Concentrations

To evaluate the dose-dependent cellular responses and establish the therapeutic window for intervention, a preliminary pilot study was conducted in which SH-SY5Y cells were screened with serial dilutions of levodopa ranging from 6.25 to 200 µM (including 6.25, 12.5, 25, 50, 100, and 200 µM) for 24 h. Cell viability was subsequently measured using the MTT assay to evaluate the safety profile and biocompatibility of each dose, thereby determining the optimal, non-toxic concentration (50 µM) (which is molecularly equivalent to approximately 20 µg/mL) required to investigate subsequent neuroprotective efficacy against rotenone-induced cytotoxicity.

Biocompatibility of Red Laser Irradiation

Similarly, to determine the safe therapeutic window for photobiomodulation, the cells were pre-screened by exposure to continuous red laser light (630–660 nm, 100 mW, 0.5 W/cm^2) at graded exposure durations (10, 20, 40, 80, 160, and 320 s) without toxin intervention, verifying optimal biocompatibility and establishing the active time frames [13,14].

Efficacy Assay Protocol (MTT Assay)

For the definitive cytoprotection experiments, following the corresponding pre-treatment windows and the subsequent 24-hour rotenone insult, MTT solution (5 mg/mL) was introduced into each well and incubated at 37 °C for 4 h to drive mitochondrial formazan crystallization. The medium was then gently aspirated, and 100 µL of DMSO was added to dissolve the purple crystals formed. The absolute optical density (OD) was measured at a detection wavelength of 570 nm using a microplate reader (800 TS, BioTek, USA). All survival scores were normalized and expressed as percentages relative to those of the untreated negative control group.

Preparation of Reagents and Solutions

Rotenone Stock and Working Solutions

The rotenone stock solution was dissolved in anhydrous dimethyl sulfoxide (DMSO) and diluted in the culture medium to the required working concentrations [12].

Red Laser PBM Calibration and Dosimetry

Photobiomodulation (PBM) procedures were performed at the Pharmacology Laboratory, College of Medicine, University of Babylon, Iraq. A calibrated continuous-wave red laser system (Dragon, China) emitting light within the wavelength range of 630–660 nm was used as the irradiation source for the samples. The laser probe was positioned perpendicular to the surface of the culture plate at a fixed distance to ensure uniform irradiation. The complete dosimetric characteristics and calibration parameters of the laser system are listed in Table 1 [14].

Table 1. Laser Photobiomodulation Dosimetric and Calibration Parameters.

Parameter	Specification / Value
Laser Light Source	Calibrated continuous-wave red laser system (Dragon, China)
Wavelength (λ)	630–660 nm (Red spectrum)
Output Power (P)	100 mW (0.1 W)
Irradiation Spot Size (Area)	0.2 cm²
Power Density (Irradiance)	0.5 W/cm² (Calculated as: 0.1 W / 0.2 cm²)
Exposure Durations (t)	10 seconds | 20 seconds | 40 seconds
Total Delivered Energy	1 Joule | 2 Joules | 4 Joules (Calculated as: P × t)
Energy Density (Fluence)	5 J/cm² | 10 J/cm² | 20 J/cm² (Calculated as: Power Density × t)
Application Mode	Continuous wave (CW), fixed perpendicularly at a constant distance

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Experimental Groups and Treatment Protocols

To systematically characterize the protective efficacy of red laser PBM pre-treatment against rotenone-induced neurotoxicity, SH-SY5Y cells were assigned to the following experimental groups:

Negative Control Group: Cells maintained under standard physiological culture conditions in complete medium without any intervention.

Rotenone (ROT) Control Group: Cells exposed to 50 µM Rotenone for 24 h to establish a neurodegenerative injury model.

ROT + L-Dopa Groups (Pre-treatment): Cells pre-incubated with levodopa at graded concentrations (25, 50, and 100 µM) for 2 h, followed by a 24-hour exposure to 50 µM rotenone, serving as the positive pharmaceutical reference arm.

ROT + Red Laser PBM Groups (Pre-treatment): Cells pre-conditioned with red laser irradiation (630–660 nm) for 10, 20, or 40 s. Following optical stimulation, the cells were incubated for 2 h before being challenged with 50 µM rotenone for a final 24-hour incubation period.

ROT + Combined Regimens (Triple Pre-treatments): Cells were pre-treated with the optimized combined configurations of levodopa and red laser PBM, designated as Triple Pre combinations (including Triple Pre, Triple Pre 2, and Triple Pre 3), followed by a 24 h exposure to 50 µM rotenone to investigate potential synergistic anti-inflammatory and antioxidant outcomes.

Rationale for Triple Predose Pairings: The specific dose combinations in the Triple Pre groups were designed based on an escalating dose-matching strategy that systematically pairs proportionally increasing concentrations of levodopa with correspondingly increasing PBM energy densities. Triple Pre 1 (25 µM + 10 s/5 J/cm²) represents the lowest pharmacological and photonic doses; Triple Pre 2 (50 µM + 20 s/10 J/cm²) combines the individually optimized doses of both modalities; and Triple Pre 3 (100 µM + 40 s/20 J/cm²) pairs the highest tested concentrations. This proportional escalation design enables systematic evaluation of dose-dependent synergistic interactions across the full therapeutic range, allowing identification of both beneficial and adverse combinatorial thresholds. This approach is consistent with factorial dose-escalation strategies commonly employed in multimodal pharmacological studies [14, 20, 21].

Triple Pre (Group 1): Cells pre-treated with 25 µM levodopa combined with a 10 s red laser PBM exposure.

Triple Pre-2 (Group 2): Cells pre-treated with 50 µM levodopa combined with a 20 s red laser PBM exposure.

Triple Pre-3 (Group 3): Cells pre-treated with 100 µM levodopa combined with 40 s red laser PBM exposure.

Biochemical and Immunological Assays

Quantification of Interleukin-1 Beta by ELISA

Intracellular neuroinflammatory signaling was monitored by quantifying the concentration of the cardinal pro-inflammatory cytokine Interleukin-1 beta (IL-1β) in the cell culture supernatants. Samples were analyzed using a commercial Human IL-1β Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Elabscience, USA) according to the manufacturer's instructions.

Assessment of Lipid Peroxidation (MDA Assay)

Oxidative structural damage to cellular lipid bilayers was quantified by evaluating malondialdehyde (MDA) accumulation in cell lysates using a colorimetric Thiobarbituric Acid Reactive Substances (TBARS) assay kit. Briefly, harvested cell lysates from all experimental cohorts were reacted with Thiobarbituric Acid (TBA) under high-temperature conditions (90–100 °C) in an acidic environment. This biochemical reaction yields a pink chromogenic complex that is proportional to the MDA mass in the sample. The absorbance profile of the isolated supernatant was evaluated spectrophotometrically at 532 nm wavelength. Final MDA values were resolved against a certified standard calibration curve, normalized to total cellular protein indices, and expressed as nanomoles per milligram of protein.

Determination of Total Antioxidant Capacity

The defensive capabilities of endogenous antioxidant enzyme pathways were tracked by determining TAOC using a ferric reducing ability colorimetric assay kit. The underlying mechanism relies on the collective ability of antioxidant enzymes in the lysate sample to reduce ferric complexes (Fe^{3+}) to ferrous forms (Fe^{2+}), which subsequently interact with a specialized dye matrix to generate a stable deep-blue chromophore. The color intensity was scanned at an absorbance of 593 nm. The quantified TAOC fractions were derived from a verified standard curve calibration and expressed as U/mg protein.

Statistical Analysis

All experimental data are expressed as mean ± Standard Error of the Mean (SEM) derived from five independent biological replicates (n = 5) per group. Statistical evaluations and significance testing were performed using GraphPad Prism software (version 9.0 or later, GraphPad Software, San Diego, California,

USA). Quantitative data from all assays (MTT absorbance values, IL-1β concentrations, MDA levels, and TAOC values) were first assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test prior to parametric analysis. Comparisons among the multiple experimental groups (including negative controls, rotenone-treated models, individual levodopa concentrations, laser PBM durations, and triple-pre-combined regimens) were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A P-value of less than 0.05 was considered statistically significant, and the levels of significance were designated as follows: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$), while ns indicated non-significant differences.

The Central Biological Ethics Committee of the College of Medicine, Babylon, approved the experimental procedures (no. 1248/14-11-2025).

Results

Cell Viability Assay (MTT Assay)

Optimization of Rotenone-Induced Cytotoxicity

SH-SY5Y cells were incubated with rotenone (0.26–80 µg/mL) for 24 h. One-way ANOVA showed highly significant differences among the groups ($F(8, 36) = 27.33$, $P < 0.0001$). As shown in Figure 1. The negative control group exhibited a mean cell viability of 100.0 ± 2.3%, which was progressively reduced in a dose-dependent manner. Dunnett's test confirmed a dose-dependent decrease in cell viability, with significant cytotoxicity observed at the following concentrations: 80 µg/mL: $P < 0.0001$ and 40 µg/mL: $P < 0.0001$ (****), and 20 µg/mL: $P = 0.0001$ (***).

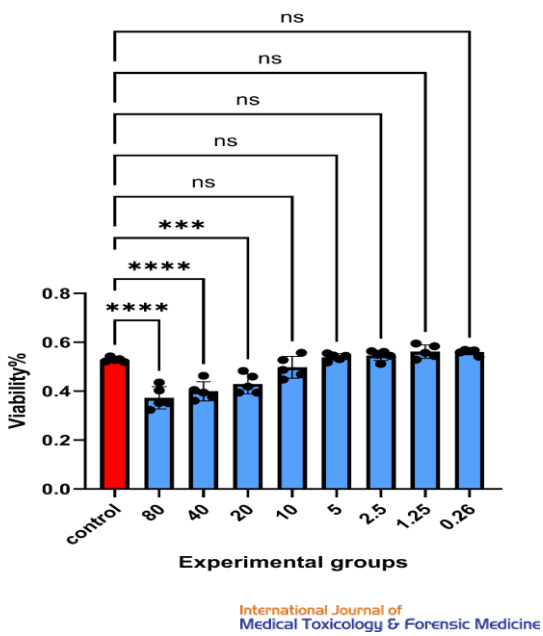

Figure 1. Dose-dependent cytotoxicity curve of Rotenone on SH-SY5Y cells after 24 hours of exposure. Data are presented as Mean ± SEM (n = 5).

Lower doses (0.26–10 µg/mL) did not induce significant cytotoxic changes ($P > 0.05$). Thus, the toxic dose profile was mapped to select the induction baseline for the subsequent experiments.

Effect of Red Laser Duration on Cell Viability

Statistical analysis revealed a significant variance in cell viability among the groups ($P = 0.0083$). Pre-treatment with a 20 s red laser PBM exposure induced a highly significant increase in cell viability compared to the rotenone control group ($P = 0.0087$), with the 20 s group achieving a mean viability of approximately 85.4 ± 4.2% compared to 62.7 ± 3.8% in the rotenone control group. Conversely, no statistically significant changes were observed following 10 s and 40 s laser exposure (10 s: 68.3 ± 5.1%, $P > 0.05$; 40 s: 65.9 ± 4.7%, $P > 0.05$). Figure 2.

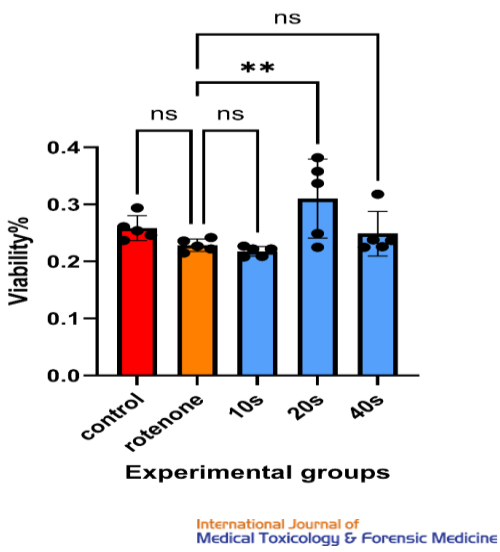

Figure 2. Effect of red laser PBM exposure durations on cellular viability (MTT assay) in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

Effect of Levodopa Concentrations on Cell Viability

The overall variance among the studied groups was not significant. Pre-treatment with individual levodopa doses showed no statistically significant differences compared to the rotenone control group. Additionally, the baseline change between the rotenone and negative control groups remained non-significant (Figure 3).

Biochemical Analysis of Neuroinflammation (IL-1\beta Levels)

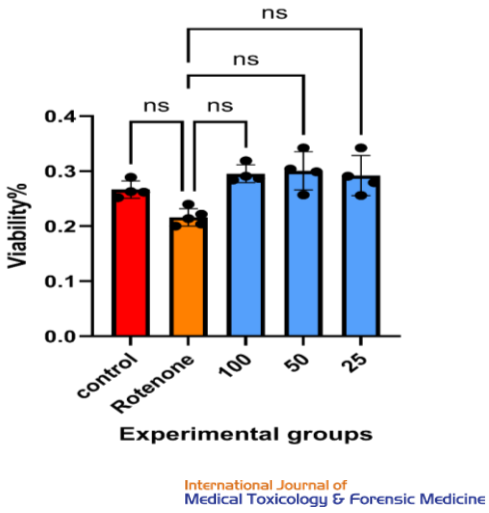

Figure 3. Effect of individual Levodopa pre-treatment concentrations on cellular viability (MTT assay) in rotenone-induced SH-SY5Y cells. Data are presented as Mean± SEM.

Effect of Individual Red Laser PBM and Levodopa Pre-treatments on IL-1β

Statistical analysis revealed a highly significant variance in IL-1β levels among the studied groups (P=0.0002), with rotenone exposure inducing a significant baseline increase compared to the negative control group. The rotenone control group exhibited IL-1β levels of approximately 245.6 ± 12.3 pg/mL compared to 98.4 ± 8.7 pg/mL in the negative control

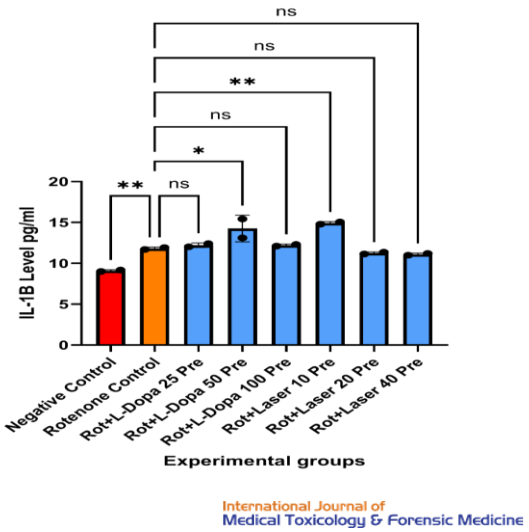

Figure 4. Effect of individual Levodopa pre-treatment concentrations and red laser PBM exposure durations on IL-1β levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean± SEM.

(P<0.001). Individual levodopa and PBM pre-treatments did not significantly alter IL-1β levels relative to the rotenone control (P>0.05 for all individual treatment groups) (Figure 4).

Effect of Combined Red Laser PBM and Levodopa Pre-treatment on IL-1β

Statistical analysis revealed a highly significant variance in IL-1β levels among the combined treatment groups (P < 0.0001), with rotenone exposure inducing a significant baseline increase compared to the negative control group (P = 0.0001). Regarding the combined therapies, pre-treatment with Triple Pre caused a significant elevation in IL-1β production compared to the rotenone control group (P = 0.0002), reaching levels of approximately 412.8 ± 18.5 pg/mL. Similarly, pre-treatment with Triple Pre 2 induced a highly significant increase in cytokine levels (P < 0.0001), with IL-1β concentrations reaching approximately 523.4 ± 22.1 pg/mL. Conversely, the alterations observed following Triple Pre 3 combined pre-treatment were not significant compared to the rotenone baseline (268.9 ± 15.4 pg/mL, P > 0.05) (Figure 5).

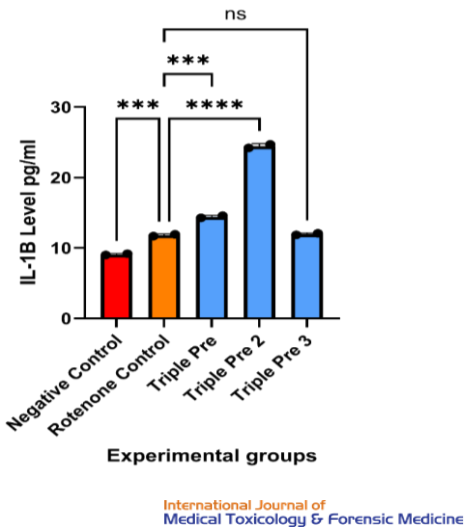

Figure 5. Effect of combined pre-treatments (Triple Pre-combinations) on IL-1β levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

Biochemical Analysis of Lipid Peroxidation (MDA Levels).

Effect of Individual Red Laser PBM and Levodopa Pre-treatments on MDA

Rotenone exposure caused a highly significant increase in MDA levels compared to that in the Negative Control (P < 0.0001). Pre-treatment with all

individual doses of levodopa (25, 50, and 100 µM) and all durations of red laser PBM (10, 20, and 40 s) significantly reduced MDA levels compared to the rotenone control group ($P < 0.0001$). The 20 s laser exposure showed the most significant protective effect ($P < 0.0001$). Figure (6).

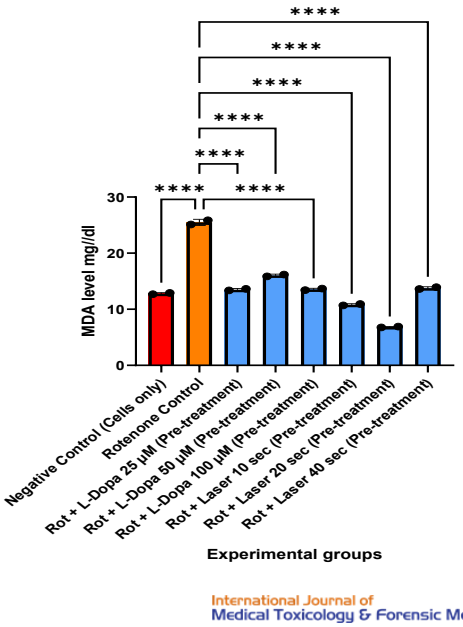

Figure 6. Effect of individual red laser PBM and Levodopa pre-treatments on MDA levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

Effect of Combined Red Laser PBM and Levodopa Pre-treatment on MDA

The combination pre-treatment protocols—comprising [L-Dopa (25 µM + Laser 10s], [L-Dopa (50 µM + Laser 20s], and [L-Dopa (100 µM + Laser 40s] exhibited a highly significant reduction in MDA concentrations compared to the toxic rotenone control group ($P < 0.0001$). Notably, the combined regimen of [L-Dopa (50 µM + Laser 20s] induced the most prominent synergistic protection, successfully restoring MDA levels close to baseline limits ($P < 0.0001$). Figure (7).

Evaluation of Total Antioxidant Capacity Levels

Effect of Individual Red Laser PBM and Levodopa Pre-treatments on TAOC

Statistical analysis confirmed a highly significant variance in TAOC levels among all groups ($P<0.0001$). Pre-treatment with all individual concentrations of levodopa (25, 50, and 100 µM) induced a highly significant upregulation in TAOC capacity compared to the rotenone control group ($P < 0.0001$). Concurrently, standalone red laser PBM pre-treatment for 20 s significantly increased TAOC levels ($P = 0.0019$), while the 10 and 40 s durations showed no significant

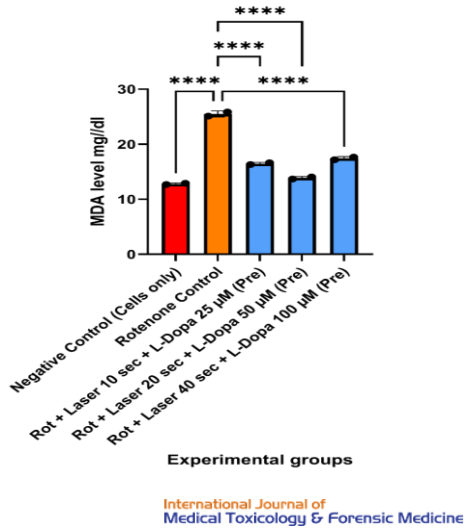

Figure 7. Effect of combined red laser PBM and Levodopa pre-treatments on MDA levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

changes ($P > 0.05$, ns) (Figure 8).

Effect of Combined Red Laser PBM and Levodopa Pre-treatment on TAOC

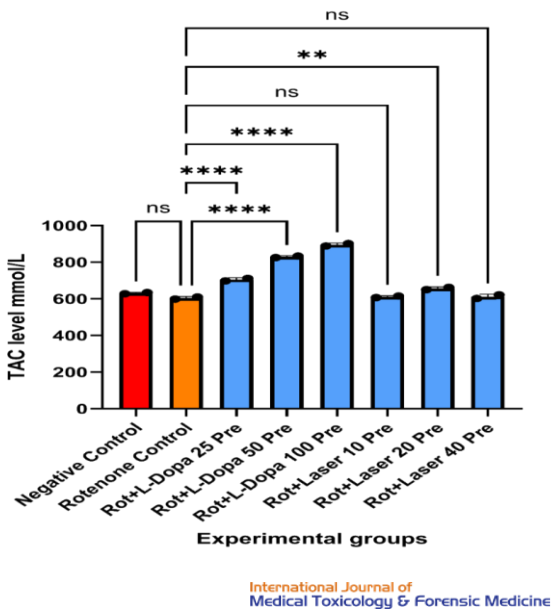

Figure 8. Effect of individual red laser PBM and Levodopa pre-treatments on TAOC levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

The combination pre-treatment protocols (Triple Pre, Triple Pre 2, and Triple Pre 3) provoked a highly significant, dose-dependent upregulation of TAOC levels compared to the rotenone control group ($P < 0.0002$ for Triple Pre; $P < 0.0001$ for Triple Pre 2 and 3). The combined regimens demonstrated a powerful synergistic effect in boosting and restoring the total intracellular antioxidant pool. Figure (9).

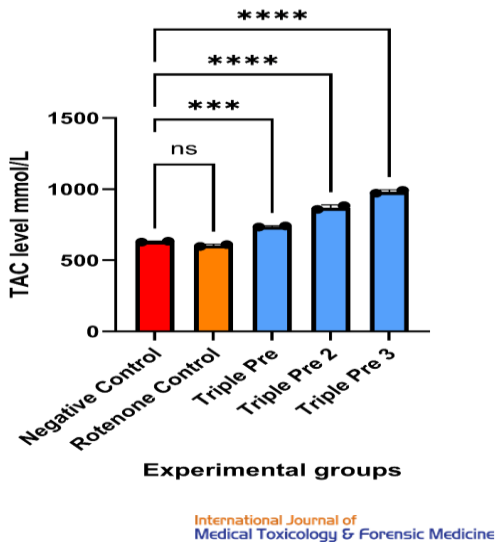

Figure 9. Effect of combined red laser PBM and Levodopa pre-treatments on TAOC levels in rotenone-induced SH-SY5Y cells. Data are presented as Mean ± SEM.

Discussion

In this study, we demonstrated that rotenone induced severe dose-dependent cytotoxicity in SH-SY5Y cells, establishing a robust in vitro model of PD. This aligns with the established literature demonstrating that rotenone selectively inhibits mitochondrial Complex I, leading to mitochondrial dysfunction, reactive oxygen species (ROS) overproduction, lipid peroxidation, and ATP depletion [15–17]. This steady state of cellular injury provides a reliable baseline for evaluating subsequent neuroprotective protocols.

Individual Levodopa pre-treatment failed to rescue cell viability, indicating that exogenous dopamine replenishment is insufficient to reverse the rotenone-induced Complex I blockade. Furthermore, dopamine auto-oxidation generates reactive quinones and free radicals, which may exacerbate oxidative stress and impair mitochondrial function independently of oxygen-dependent mechanisms, particularly in an already compromised mitochondrial environment [18, 19].

In sharp contrast, the photobiomodulation (PBM) outcomes demonstrated a clear time-dependent response. While the 10-second and 40-second laser durations were non-significant, pre-treatment with a 20-second red laser exposure induced a highly significant upregulation in metabolic viability scores ($P = 0.0087$). This prominent outcome highlights the classic biphasic dose-response phenomenon typical of PBM, where cellular biostimulation only occurs within a strict, optimal energy density threshold, whereas under- or over-exposure yields no benefit, or can even be inhibitory [20, 21]. Huang et al. extensively characterized this Arndt-Schulz biphasic dose-response curve in low-level light therapy, demonstrating that low levels of light have a significantly superior effect on stimulating and repairing tissues compared to higher levels, which may produce inhibitory outcomes [20]. Sharma et al., further confirmed this biphasic pattern specifically in neuronal cells, reporting that optimal energy densities typically range between 3 and 10 J/cm², with supra-threshold doses failing to elicit neuroprotection [21]. At 20 seconds (2 J, 10 J/cm²), the 630–660 nm red light efficiently primes cellular metabolism. This specific photon dose is absorbed by cytochrome c oxidase (Complex IV) in the mitochondrial inner membrane, accelerating electron transfer, restoring mitochondrial membrane potential, and triggering a critical surge in ATP synthesis [22, 23]. Chang et al., demonstrated that photobiomodulation significantly enhances mitochondrial membrane potential and ATP production in neural cells through direct photon absorption by cytochrome c oxidase [22]. This mechanism has been further validated by Shen et al., who reported in their comprehensive scoping review that PBM activates multiple mitochondrial signalling pathways, resulting in enhanced bioenergetics and upregulation of antioxidant defenses [23].

Crucially, the present study evaluated the impact of these treatments on lipid peroxidation and endogenous antioxidant defences. Rotenone exposure caused a highly significant increase in MDA levels and a depletion of the TAOC. Individual red laser PBM, particularly at the optimal 20-second exposure, significantly reduced MDA concentrations ($P = 0.0002$) and restored TAOC ($P = 0.0004$). These findings are strongly supported by contemporary research demonstrating that PBM effectively mitigates oxidative stress by upregulating antioxidant enzymes and reducing lipid peroxidation markers in neuronal cells [24, 25]. Salehpour et al. reported that transcranial near-infrared photobiomodulation noticeably reduced oxidative stress in hippocampal neurons, as measured by lipid peroxidation markers, and simultaneously enhanced TAOC [24]. Remarkably, Rossato et al. demonstrated that LED irradiation at 660 nm was sufficient to improve mitochondrial activity in SH-SY5Y cells exposed to oxidative stress, providing direct evidence that red-spectrum PBM confers cytoprotection in the same neuroblastoma model employed in the present investigation [25]. The ability of PBM to shift the cellular redox state towards a protective antioxidant profile is a fundamental mechanism underlying its neuroprotective efficacy against mitochondrial toxins [26]. Li et al. confirmed that photobiomodulation provides neuroprotection

specifically by targeting neuronal mitochondria, alleviating mitochondrial fission imbalance-induced neuronal apoptosis, and thereby restoring cellular bioenergetic homeostasis [26]. Additionally, all individual levodopa concentrations (25, 50, and 100 µM) significantly reduced MDA levels and restored TAOC, indicating that despite its failure to rescue cell viability, levodopa retains intrinsic antioxidant properties at the biochemical level, likely through its catechol moiety acting as a direct free radical scavenger [19].

A pivotal and novel aspect of this investigation was the evaluation of combined therapeutic regimens (Triple Pre-treatments). The combination protocols provoked a highly significant, dose-dependent upregulation in TAOC and a marked reduction in MDA levels, with the [Ldopa 50 µM + Laser 20s] regimen inducing the most prominent synergistic protection against oxidative damage ($P < 0.0001$). However, the evaluation of neuroinflammation, assessed via IL-1β levels, revealed complex and paradoxical interactions. While individual optimal PBM is generally recognized for its anti-inflammatory properties [27, 28], specific combined regimens (Triple Pre 1 and Triple Pre 2) triggered a synergistic surge in IL-1β production ($P = 0.0002$ and $P < 0.0001$, respectively). Hamblin comprehensively reviewed the anti-inflammatory mechanisms of PBM, demonstrating that it can reduce markers of the M1 macrophage phenotype and decrease pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, in various disease models [27]. Cardoso et al. further confirmed that transcranial PBM modulates the neuroinflammatory profile by reducing pro-inflammatory cytokine expression and enhancing anti-inflammatory signaling in aged brain tissue [28]. Therefore, the paradoxical pro-inflammatory response observed in our combined treatment groups represents a novel and clinically significant finding that warrants careful mechanistic interpretation.

The paradoxical pro-inflammatory synergy observed in the combined regimens is hypothesized to be driven by hypermetabolic cross-talk. While PBM accelerates cellular metabolism and ROS signaling to protective levels, this enhanced activity may inadvertently accelerate the oxidation of exogenous levodopa into reactive dopamine quinones [29]. The accumulation of these reactive species can activate the NLRP3 inflammasome pathway, driving the robust processing and release of mature IL-1β [30–33]. Thus, the convergence of PBM-enhanced bioenergetics with levodopa auto-oxidation may create an inflammatory microenvironment that overwhelms the intrinsic anti-inflammatory capacity of the PBM. This mechanistic interpretation remains speculative, as direct measurement of dopamine quinone levels or NLRP3 activation was not performed in the current study; future investigations employing targeted metabolomics and inflammasome-specific assays are warranted to confirm this proposed mechanism.

Interestingly, the Triple Pre 3 configuration (100 µM Levodopa + 40s Laser) maintained cytokine homeostasis, showing no significant elevation in IL-1β compared to the rotenone baseline. This observation suggests that higher levodopa concentrations paired with longer laser durations (which deliver supra-threshold energy densities of 20 J/cm²) may suppress the hypermetabolic inflammatory window, paradoxically achieving a safer inflammatory profile[20]. According to the Arndt-Schulz model, the 40-second exposure falls within the inhibitory phase of the biphasic curve, potentially attenuating the metabolic acceleration that drives levodopa auto-oxidation at lower stimulatory doses [20, 21]. This finding has profound translational implications, as it suggests that careful dosimetric calibration can identify safe therapeutic windows for combined PBM-pharmacological interventions in patients with Parkinson's disease.

These findings have significant translational implications for the emerging field of multimodal neuroprotective strategies. Farazi et al. conducted a comprehensive systematic review of PBM combination therapies in neurological disorders, concluding that most combination approaches produce synergistic effects, leading to superior outcomes compared to monotherapies [32]. However, the present study provides a critical caveat: the concurrent administration of PBM with exogenous catecholamines necessitates rigorous dosimetric thresholding to avoid paradoxical pro-inflammatory effects. Harnessing the synergistic bioenergetic and antioxidant benefits while avoiding severe pro-inflammatory cascades is paramount for the development of safe and effective multimodal interventions in Parkinson's disease. Future investigations should explore the temporal dynamics of this inflammatory response, assess whether sequential rather than concurrent administration mitigates pro-inflammatory synergy, and validate these findings in differentiated dopaminergic neuronal models and in vivo Parkinson's disease paradigms.

Study limitations

Several limitations of this study should be acknowledged. First, the use of undifferentiated SH-SY5Y cells, while widely accepted as a dopaminergic cell model, does not fully recapitulate the phenotypic and functional characteristics of mature dopaminergic neurons in vivo. Undifferentiated SH-SY5Y cells exhibit a mixed neuronal-epithelial phenotype and may respond differently to pharmacological and photonic stimuli compared to terminally differentiated

dopaminergic neurons, which express higher levels of dopamine transporters, D2 receptors, and mature synaptic machinery. Differentiation with retinoic acid and/or brain-derived neurotrophic factor (BDNF) would enhance the translational relevance of future studies. Second, the proposed mechanistic interpretation involving dopamine quinone-mediated NLRP3 inflammasome activation remains inferential, as direct quantification of dopamine quinones, aminochrome intermediates, or caspase-1 cleavage was not performed. Third, the in vitro monoculture system lacks the complex multicellular interactions (e.g., neuron-glia cross-talk, blood-brain barrier dynamics, and systemic pharmacokinetics) that influence therapeutic outcomes in vivo. Fourth, the study employed a pre-treatment paradigm, which may not directly translate to clinical scenarios where patients receive interventions after disease onset. Finally, while five biological replicates provide adequate statistical power for the observed effect sizes, larger sample sizes and independent experimental repetitions across different cell passages would further strengthen the reproducibility of these findings. These limitations underscore the necessity for validation in differentiated neuronal models, primary dopaminergic cultures, co-culture systems incorporating glial cells, and ultimately in vivo Parkinson's disease animal models.

Conclusion

This study demonstrates that red laser photobiomodulation at an optimal 20 s exposure (2 J, 10 J/cm²) confers potent biphasic neuroprotection against rotenone-induced cytotoxicity by restoring mitochondrial bioenergetics and fortifying endogenous antioxidant defenses in SH-SY5Y cells. The strict dose-dependency observed, where sub-threshold and supra-threshold exposures failed to elicit protection, validates the Arndt-Schulz biphasic dose-response model for PBM in neurodegeneration. Critically, while individual PBM effectively mitigated oxidative stress and lipid peroxidation, its concurrent administration with exogenous levodopa at specific dose combinations paradoxically triggered massive pro-inflammatory cascades via catecholamine auto-oxidation under laser-enhanced metabolic conditions. This finding has significant translational implications: future combinatorial neuroprotective strategies for Parkinson's disease must incorporate rigorous dosimetric thresholding to harness synergistic antioxidant benefits while avoiding the hypermetabolic inflammatory window. The identification of Triple Pre 3 as a configuration that maintains cytokine homeostasis suggests that higher levodopa concentrations paired with longer laser durations may paradoxically achieve safer inflammatory profiles, warranting further investigation in animal models and clinical settings.

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Conflicts of Interest

The authors report there are no competing interests to declare.

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