Effects of Light Emitting Diode and Low-intensity Light on the immunological process in a model of Parkinson's disease

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Abstract

Parkinson's disease (PD) is characterized primarily by the loss of dopaminergic neurons in the *substantia nigra* and appearance of alpha-synuclein aggregates in Lewy bodies. The neuroinflammation in Parkinson's disease is associated with activation of microglia, the participation of inflammatory cytokines, and systemic activation of natural killer cells. Evidence suggests that several inflammatory cytokines are enhanced in the brain and blood of patients presenting with Parkinson's disease. In addition, other studies have suggested that Light Emitting Diode (LED) and Low-intensity Light (Laser) hold potential for improving neuronal cell function in patients with Parkinson's Disease. This study investigated the influence of LED and Laser on inflammatory processes caused by an electrolytic lesion of the *substantia nigra* in an experimental model of Parkinson's disease. Sixty Wistar rats were divided into three experimental groups (LED, Laser, and Control). An electrode was placed in the cortex for PD induction that was evaluated for motor conditions after 30 days in an Open Field. Cytokines levels were analyzed by flow cytometry using the BD Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit. Serum cytokine concentrations (IL-4, IL-6, IL-10, TNF-α, IFN-γ) were significantly different in each group. When compared with other groups, the concentrations of interferon and IL-2 were higher in the LED and Laser groups, respectively. TNF-α showed lower concentrations in the LED and Laser groups when compared with the Control group. The LED and Laser actions on the central nervous system in an animal model with lesions presenting neurodegeneration and persistent inflammation, such as in Parkinson's disease, show significant effects for the treatment and prevention of neurodegeneration caused by pro-inflammatory cytokines.

Keywords: Parkinson's disease, cytokines, LED, Laser

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1. Introduction

Parkinson's disease (PD) is considered the second most common senile neurodegenerative disease of the elderly, affecting about 4% of people over 80 years of age, including men and women of different races and social classes [1]. It is characterized by the progressive loss of dopaminergic neurons in the nigrostriatal pathway and is associated with motor symptoms such as rigidity, akinesia, bradykinesia, reduced postural reflexes, and tremors. Depression, anxiety, and insomnia are among some its comorbidities [2].

The exact mechanisms that lead to the death of dopaminergic neurons in PD are still unclear. Mitochondrial dysfunction, oxidative stress, protein structural changes, and inflammatory processes related to immunity are some factors that can lead to cell dysfunction and consequent neuronal death [3].

Previous studies reported elevated levels of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-alpha (TGF-α), in dopaminergic neurons in the striatum of patients with PD. Parkinsonian signs and neuronal loss in the nervous system were observed in an immunized PD animal model, reinforcing the importance of studying the disease's immunological mechanisms [4-6].

Therapies that use low-intensity Light Emitting Diode (LED) are presented as inexpensive and advantageous alternatives to treatments compared to the use of Laser [7] because they show similar results [8]. It is well established in the literature that both Laser (low-intensity Laser therapy) [9,10] and LED [11,12] therapies have positive effects during the process of tissue repair, including the modulation of pro-inflammatory cytokines [13,14] and increased production of anti-inflammatory activity of cytokines.

The Laser therapy used in tissues and cells is not based on heating, i.e., the energy of absorbed photons is not transformed to heat but to photochemical, photophysical, and/or photobiological effects [15]. Also according to the authors, when the laser light interacts with cells and tissues in the appropriate dose, certain cell functions can be stimulated, such as

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stimulation of lymphocytes, mast cell activation, increase in mitochondrial ATP production, and proliferation of various types of cells, thus promoting, anti-inflammatory effects (Rocha Junir et al., 2007).

This study evaluated the influence of LED and Laser applications on the inflammatory process caused in a PD animal model.

2. Methodology

2.1. Sample

Sixty albino Wistar rats (*Rattus norvegicus*), weighing between 200g and 250g from the Pontifical Catholic University of Paraná (PUC-PR), were used in the study; they were separated into groups and housed in pairs in acrylic cages with access to water and food (*ad libitum*), under a 12 hours light/dark cycle, and room temperature of 23 ± 1°C.

The animals were divided into three groups:

- Control Group (C): composed of 20 animals with induced lesion in the substantia nigra compact and irradiated.

- LED Group (LED): composed of 20 animals being induced injury in the compact substantia nigra and treated for seven days with euthanasia on the eighth day.

- Laser Group (Laser): composed of 20 animals being induced injury in the compact substantia nigra and treated for seven days with euthanasia on the eighth day.

The experiments were conducted at the Laboratory of Neuroanatomy and Neurophysiology of the CEDETEG Campus from the Midwest State University (Unicentro) and Molecular Biology Laboratory. The ethics committee on animal use of Unicentro approved of the study (protocol number 023/2015).

2.2. Surgical procedure

The animals were intraperitoneally anesthetized with 80mg/kg ketamine (Ketamine, 10ml vial) and 15 mg/kg xylazine (Dopaser, 10ml vial). Once anesthetized, they were taken to a stereotaxic apparatus (David Kopf, USA) where their heads were stabilized by the temporal inner ear and upper incisors. A copper wire number 34 electrode was implanted in the cortex and directed to the midbrain in the compact part of the *substantia nigra*

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bilateral following the anteroposterior (AP=-2.70), medium lateral (ML=±2.60), and dorsoventral (DV=4.60) coordinates and taking bregma as a reference with the lambdoid and bregmatic sutures in the same horizontal plane as Paxinos and Franklin [16] (Figure 1).

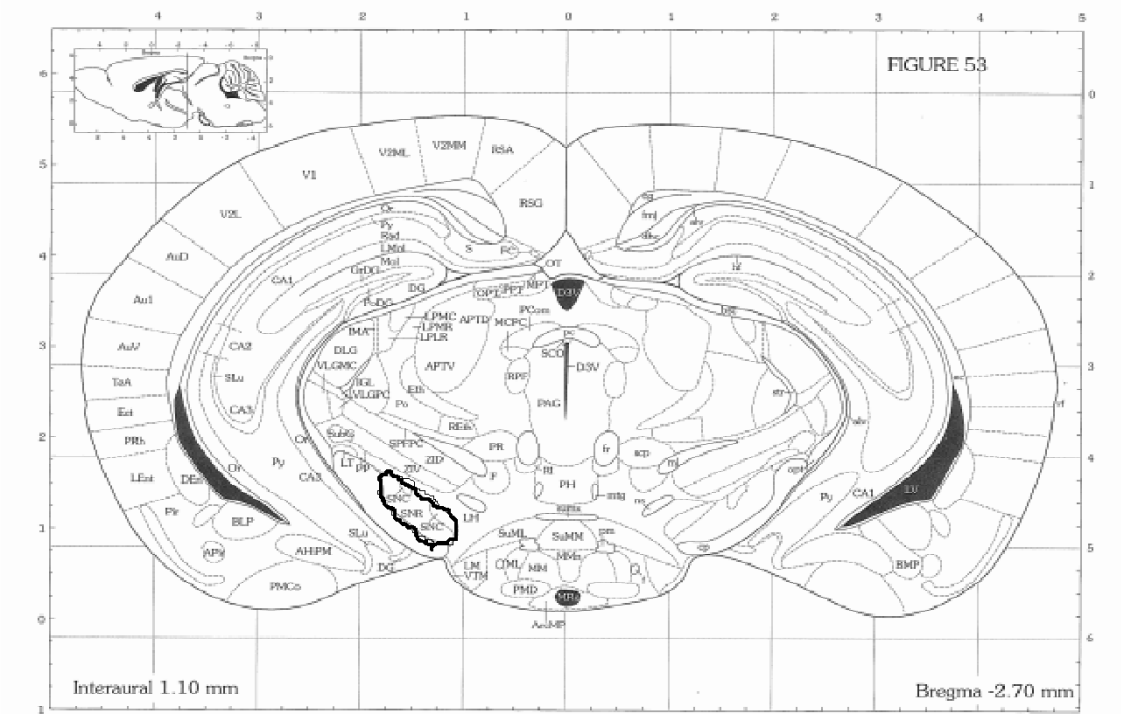

Figure 1: shows the coordinates for the realization of the lesion substantia nigra pars compacta.

2.3. Induction of PD and verification of motor condition

Bilateral microinjections of 15 µg of 6-OHDA (dissolved in 3 µl of 0.2% ascorbic acid saline solution) into the substantia nigra (SN) using a10 µl Hamilton syringe. The infusion rate was 1 µl/min, and the injection syringe was kept in place for a further 5 min after injection for complete absorption of the toxin. Animals were followed up for 30 days after treatment to induce lesions [17]. Each animal was individually placed in an arena (Open Field) for the analysis of motor behavior. Animals that remained in a given quadrant for a 5 minutes period were included in the study sample.

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2.4. Experimental groups/ Interventions

Animals subjected to lesion induction in the compact portion of the *substantia nigra* were divided into three groups of 20 animals each. Animals began receiving treatment 30 days after lesion induction. The groups were: Control Group (GC), which received no treatment; LED group (LED) treated with LED 627 nm, 4 J/cm^2 to 70 mW [27] in the right cervical region, once daily for 57 seconds; and Laser Group (Laser) treated with 630 nm, 4J/cm^2, 45mW, for 88 seconds [18, 19]. They were irradiated from a single point in the cervical region of the common carotid artery for seven days.

The choice for two types of equipment (laser and LED), but with the same wavelength, was to check the influence of the difference in coherence that has biomodulation.

2.5. Flow Cytometry

One milliliter of blood was collected from each rat. Blood was incubated in a water bath for 15 minutes and centrifuged at 300g for 5 minutes at 18^{o}C. After centrifugation, the supernatant (serum) was stained for detection of IL-2, IL-4, IL-6, TNF-α, and interferon-gamma (IFN-γ) using the BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (Becton Dickinson, USA), according to the manufacturer's instructions and analyzed in the BD™ Accuri C6 Flow Cytometer (Becton Dickinson, USA), with 10 µl of each reagent being added to each sample. After this procedure was placed in a 1.5 ml eppendorf, 50 µl beads, 50 µl sample (serum), 50 µl detection reagent. The tubes were placed in the dark for two hours. After that, 1 ml of water was added and centrifuged at 2000 RPM, 4 degrees Celsius, for 5 minutes. Thereafter, the supernatant was removed and 300 ul of water was again added.

The theoretical detection limit for each cytokine using the BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit is defined as the concentration corresponding to two standard deviations above the fluorescence average of 30 negative control replicates (0 pg/ml); these limits were: IL-2 = 0.1 pg/ml; IL-6 = 1.4 pg/ml; IL-10 = 16.8 pg/ml; IL-4 = 0.03 pg/ml, IFN-γ = 0.5 pg/ml and TNF-α = 0.9

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pg/ml. The reading on the cytometer was manually performed through the acquisition of 10,000 events from each sample. The flow cytometry data were analyzed in the FCap 3.0 Array software (Becton Dickinson, USA) and results were plotted in graphs showing averages and standard deviations.

2.6. Euthanasia

The animals were anesthetized with 80 mg/kg ketamine and 15 mg/kg xylazine and euthanized with an intraperitoneal injection of a lethal dose of thiopental 100 mg/kg.

2.7. Statistical analysis

The Gaussian distribution of the data was verified by the Shapiro-Wilk normality test. The sample was analyzed using the Kruskal-Willis test with the Dunn's post-test.

3. Results

In figure 2 we noted the average values in relation to the motor behavior of the animal before and after surgery to damage the substantia nigra pars compacta. We noticed a statistical difference between the groups before and after the injury with a value of p = 0.0005, showing a decrease of displacement, the animals remain more static than before the injury.

The present study evaluated the effect of LED and Laser on serum inflammatory cytokines of rats with induced Parkinson's disease in an experimental model.

Figure 3 shows the average and standard deviation of levels of IL-2 (A), IL-6 (B), IL-4 (C), IL-10 (D), TNF-α (E), and IFN-γ (F); values among the groups were not statistically significant. Figure A shows that the IL-2 concentrations were higher in the Laser group (18.01±13.65 pg/ml) compared to the GC group (p=0.03). Figure B shows that the IL-6 concentrations were higher in the LED group (17.87±3.20 pg/ml) than in the LED group, however, differences were not significant (p=0.076). Figures E and F show that the concentrations of IL-4 and IL-10, respectively, were higher in the Laser group than in the LED group. However differences (p=0.214 for IL-4 and p=0.611 for IL-10) were not statistically significant: IL-4 concentrations of 4.64±4.35 pg/ml for GC, 4.77±1.65 pg/ml for LED, and 20.43±20.30 pg/ml for Laser; and IL-10

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concentrations of 12.10±7.64 pg/ml for GC, 18.96±4.07 pg/ml for LED, and 23.07±8.69 pg/ml for Laser. Figure C shows TNF-α at lower concentrations in the Laser and LED groups (19.00±14.37 pg/ml and 8.12±4.04 pg/ml in the LED and Laser groups, respectively) when compared to the GC group (24.00±10.00 pg/ml, p=0.074). Figure D shows higher IFN-γ concentrations in the LED group (3.04±3.20 pg/ml) when compared to other groups (0.82±0.69 pg/ml in the GC and 0.32±0.05 pg/ml in the Laser group, p=0.0006).

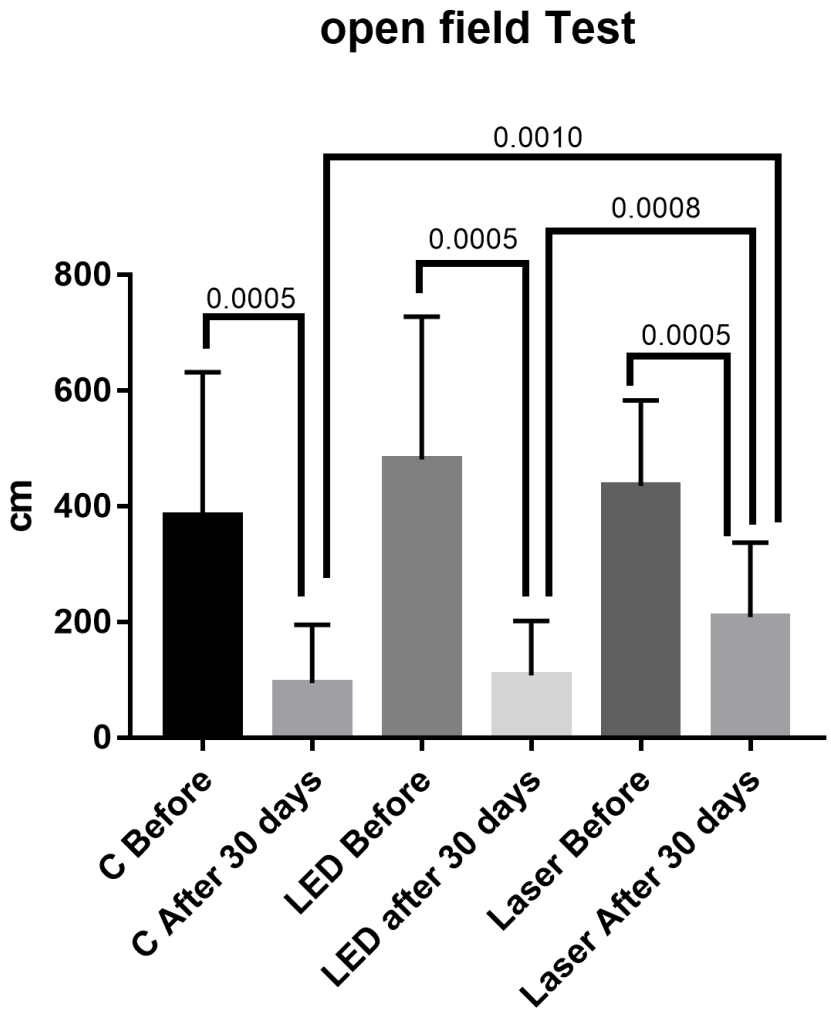

Figure 2: the open field test before the injury and after injury analyzing the animals' displacement between the groups and the displacement in the open field was smaller with siginifativo value of p <0.05.

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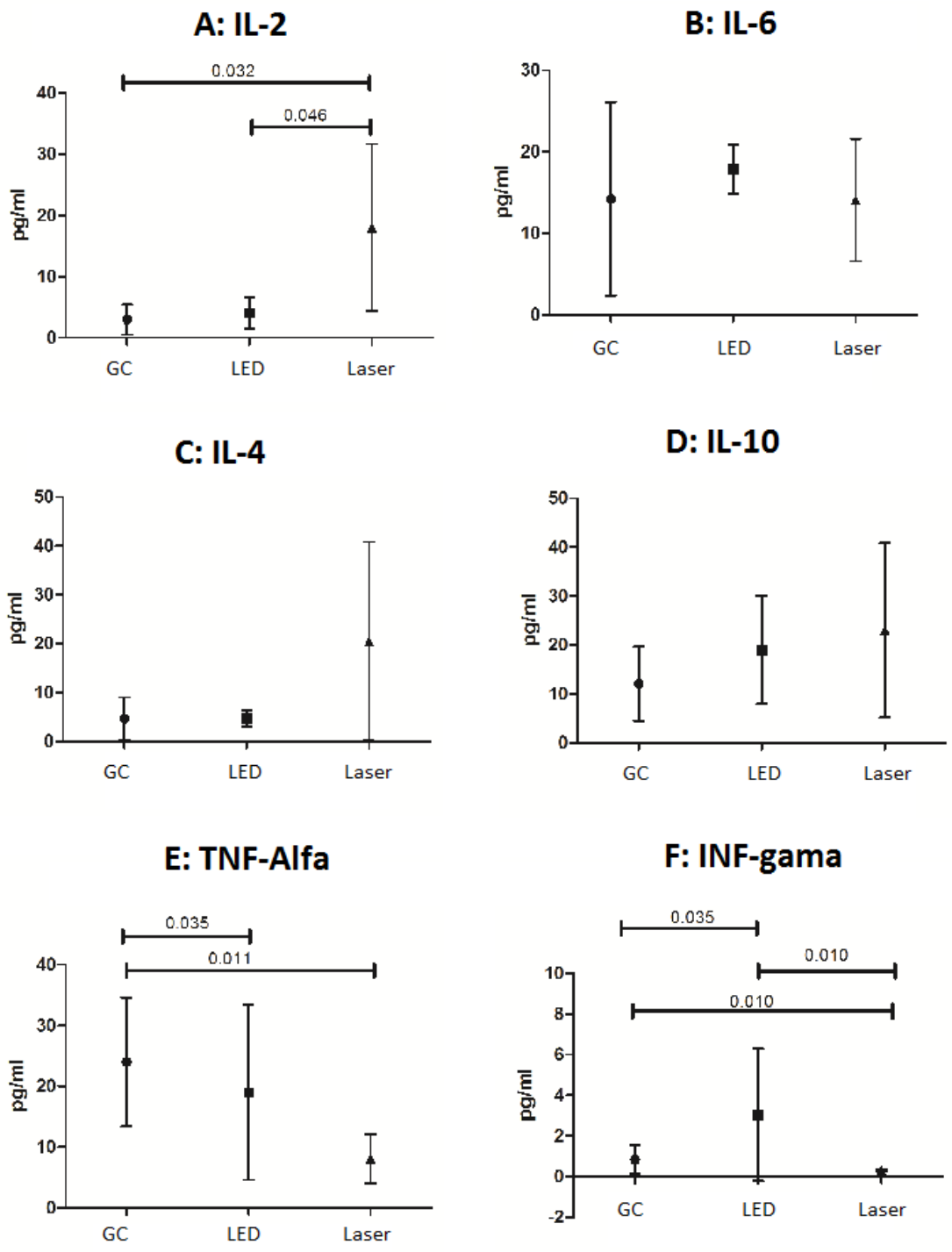

Figure 3: Averages and standard deviation (vertical bars) of cytokines values in the Control, LED, and Laser groups (pg/ml). Horizontal bars showed a significant difference between the experimental groups and control group according to the Dunn's post-test analysis. Kruskal-Wallis test with Dunn's post-test at $p<0.05$.

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4. Discussion

Neuroinflammation in the CNS has been closely associated with the pathogenesis of acute and chronic neurodegenerative diseases including Parkinson's disease. This process involves the activation of macrophages resident in the brain, which release neurotoxic and pro-inflammatory factors, including cytokines, free radicals, nitric oxide, and eicosanoids, that can damage neurons and glial cells [19]. Cytokines are groups of proteins produced in response to some trauma or antigens that mediate and regulate immune and inflammatory responses [20].

The present study verified the pro/anti-inflammatory cytokine responses to the application of low-intensity light treatment in a model of induced Parkinson's disease based on the knowledge that low-intensity light promotes anti-inflammatory activity through photo-biomodulation. Six cytokines, four pro-inflammatory and two anti-inflammatories, were quantified in peripheral blood.

We studied the effects of low-intensity Laser on the immunity of rats. The study used the HeNe Laser (632.8 nm, 0.2 mW/cm^2) irradiated for 60 seconds on the thymus and found that there was an increase in IL-2 concentration twenty-four hours after a single application [21].

Our results showed a significant increase in IL-2 in rats after a seven-day irradiation with a low-intensity Laser when compared to IL-2 concentrations in control rats and rats in the LED group, corroborating the findings of Novoselova et al.. The LED group shows no significant difference when compared to the control group.

The IL-6 effectively participates in immune response regulation, being related to reactions in the acute phase and hematopoiesis, and quickly induced in inflammatory processes [22].

The effects of low-intensity Laser (660 nm) and LED (640 nm) on the immune response of rats submitted to an acute inflammation process in the knees. The study reports that a higher concentration of IL-6 was observed in the

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group treated with LED at the dose of 2.5 J/cm^2, compared to the other groups, and immediately at 1 and 2 hours following the induction of inflammation [23].

In this study, the average values of IL-6 in the LED group were higher than that of other two groups. Therefore, the application of LED influences the activation of microglia and processes of macrophages stimulation, which releases cytokines. The increase in IL-6 can also result from the stimulation of other cytokines, such as TNF-α and INF-γ, which also showed high levels in the LED group. TNF-α and INF-γ were observed at significantly low levels in the Laser group.

The effect of low-intensity Laser on experimental models of cryogenic CNS lesions and observed that the Laser decreased the concentration of pro-inflammatory cytokines, such as TNF-α, leading to a reduction in post-lesion tissue loss [24].

Decreased TNF-α were observed, demonstrating the effectiveness of the Laser therapy on inflammation and corroborating the results reported by Moreira.

In vitro tests on human blood, reported that the application of Laser (340 and 480 nm) produces a beneficial effect on irradiated blood tissue showing decreased levels of pro-inflammatory cytokines (TNF-α and IFN-γ) and contributing to the resolution of inflammatory processes. Similar results were found in this study showing that the decreased levels of these cytokines had significant benefits in relation to the non-irradiated group [19].

LED and Laser irradiation promoted an increase in the levels of anti-inflammatory cytokine IL-10, demonstrating the anti-inflammatory action of both methods. This Interleukin inhibits other pro-inflammatory interleukins, such as TNF, INF-γ, and IL-2.

The IL-10 and IL-4 inhibit the production of TNF-α by monocytes. These authors investigated the effects of IL-10 and IL-4 on the cell surface and the release of TNF-R by human monocytes to determine if these cytokines modulated the TNF-α activity and concluded that IL-4 suppressed the release of TNF-R, and IL-10 reduced the TNF pro-inflammatory

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mechanisms. Thus, it is believed that IL-10 can be used to treat diseases that exhibit high expression of TNF-α [25].

The results of this study highlight the Th1/Th2 mechanisms involving interleukins demonstrating that the application of both LED and Laser influences the activation of anti-inflammatory mechanisms, which are associated with the stimulation of the immune system.

The levels of IL-1β, IL-2, IL-4, IL-6, EGF, and TGF-α in ventricular cerebrospinal fluid (VCSF) of non-parkinsonian, juvenile parkinsonian (JP), and Parkinson's disease (PD) patients through immune assays of highly sensitive enzymes. The concentrations of IL-1β, IL-2, IL-4, and TGF-α in the VCSF were higher in the JP group than in controls ($p < 0.05$); the IL-2 and IL-6 concentrations were higher in PD patients compared to the control group ($p < 0.05$). Thus, it was observed that the levels of these proteins were high in the dopaminergic region of individuals with PD. Alterations in the levels of cytokines in the VCSF may indicate changes in these proteins in the brain; these increases might trigger a compensatory response during the neurodegeneration in PD or JP patients. It is concluded that the increase in cytokine levels may contribute to a compensatory response and become the initial cause of neurodegeneration [26].

In the present study, increased levels of anti-inflammatory cytokines were observed, which in turn inhibited the neurodegeneration caused by IL-6 that was also detected at high levels in this study. Although rat brains were irradiated with LED and Laser for a short period of 7 days in this study, the resulting activation of these cytokines led to balance in the Th1/Th2 system.

The microglia presents a relevant pathogenic role in neurodegenerative diseases mediated by immune factors. Choi used co-cultures of microglia and neuronal cells immunized with IFN-γ and observed that liposaccharides and TNF-α triggered neuronal lesion due to an impaired absorption of gamma-aminobutyric acid and neuronal loss through a decrease in nitric oxide. The dose-dependent pretreatment of the co-cultures with IL-4 prevented neuronal cell lesions induced by activated microglia.

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IL-4 acts as a neuroprotector inhibiting IFN-γ and decreasing the synthesis of TNF-α and nitric oxide [27].

IL-4 was detected in high levels in the samples treated with Laser, corroborating the results reported by Choi in relation to neurodegeneration control in the presence of high levels of this protein.

5. Conclusion

The actions of LED and Laser on the central nervous system, on lesions presenting neurodegeneration and persistent inflammation, such as in the case of Parkinson's disease, show significant effects for the treatment and prevention of neurodegeneration caused by pro-inflammatory cytokines.

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