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Exploring the Neuroprotective Effects of Nicotine Against MPTP-Induced Neuronal Damage in Mice: Insights into Antioxidant System

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Abstract Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, following Alzheimer's disease, impacting approximately 1% of individuals aged 60 and older. It is characterized by the degeneration of dopaminergic neurons in the SNpc, resulting in both motor and non-motor symptoms. Current treatments are limited, and PD's molecular basis involves alpha-synuclein aggregation, ubiquitination dysregulation, and oxidative stress. Nicotine, found in tobacco, has been associated with reduced PD risk in epidemiological studies and shown neuroprotective effects in animal models. However, its impact on oxidative stress remains unclear. A study involving 160 albino laboratory mice investigated nicotine's effects in an MPTP-induced PD model. The mice were divided into four groups: control, nicotine, MPTP, and MPTP + nicotine. The study assessed behavioral tests, dopamine levels in the SNpc, and antioxidant system activity. Nicotine improved motor function, increased dopamine levels, and reduced oxidative stress markers in the PD model. Importantly, it selectively enhanced the activity of antioxidant enzymes, superoxide dismutase, and catalase, in PD-affected mice without affecting healthy animals. Nicotine demonstrates neuroprotective effects in an MPTP-induced PD model, ameliorating motor deficits, increasing dopamine levels, and reducing oxidative stress. It enhances the activity of key antioxidant enzymes in the substantia nigra, suggesting potential therapeutic avenues for PD treatment. However, further research is needed, including human studies and a deeper understanding of nicotine's impact on oxidative stress. These results contribute to our understanding of PD pathogenesis and offer insights into novel therapeutic approaches for this debilitating disorder. They also raise questions about the differential effects of nicotine in healthy individuals and those with PD, highlighting the need for targeted interventions in neurodegenerative diseases.

Keywords Parkinson's Disease, Nicotine, Antioxidant System, MPTP, Neuroprotection

1. Introduction

Parkinson's disease (PD) represents a progressive neurodegenerative ailment characterized by the deterioration of dopaminergic neurons within the Substantia nigra pars compacta (SNpc). This process precipitates a spectrum of motor symptoms, including akinesia, rigidity, and tremors, alongside non-motor manifestations such as depression and cognitive impairment [1,2]. Regrettably, despite strides in comprehending PD, it remains devoid of a cure, with

disease progression primarily managed through the administration of the dopamine precursor, L-Dopa. It is pertinent to acknowledge projections by Dorsey et al., estimating that by the year 2040, PD will afflict an estimated 12 million individuals [3]. Given the intricate nature of the underlying pathophysiological mechanisms, it becomes imperative to rigorously investigate these processes in pursuit of potential therapeutic interventions. The molecular underpinnings of PD are intricately tied to several pivotal phenomena: 1. the abnormal aggregation of alpha-synuclein, culminating in the formation of Lewy bodies; 2. dysregulation of the ubiquitination process; and 3. the emergence of oxidative stress as a consequence of mitochondrial dysfunction, leading to the accumulation of damaged proteins and the generation of reactive oxygen and nitrogen species. Collectively, these factors contribute significantly to the degeneration and ultimate demise of dopaminergic neurons in the SNpc [4].

Several compounds are known that have a positive protective effect in neurodegenerative diseases, including Parkinson's disease. These substances effectively protect nerve cells from damage and at the same time reduce the likelihood of damage to these cells during various neurodegenerative changes. These compounds are characterized by a specific mechanism of action aimed at protecting nerve cells and restoring their functionality. Such compounds include, for example, coenzyme Q, lipoic acid, acetyl-L-carnitine, levodopa, and others [5].

Nicotine, the main alkaloid found in tobacco, constitutes approximately 0.6-3.0% of its dry weight. Remarkably, epidemiological investigations have indicated that individuals who smoke cigarettes exhibit a reduced mortality rate in Parkinson's disease when compared to non-smokers, thereby suggesting a potential influence of nicotine on the course of this debilitating disorder [6-8]. Furthermore, the neuroprotective properties of nicotine have been substantiated through diverse in vivo studies [9-11]. These investigations not only underscore the advantageous impact of nicotine in animal models with Parkinsonism but also elucidate various signaling pathways implicated in this phenomenon. Notably, these pathways include the downregulation of SIRT-6, the inhibition of PARP-1 and caspase-3 cleavage, as well as the modulation of the p-JNK/JNK and p-ERK/ERK ratios. It is conceivable that the neuroprotective efficacy of nicotine extends beyond these pathways, encompassing other cellular regulatory mechanisms. Of particular interest is the inquiry into whether nicotine's action is associated with a reduction in oxidative stress, a paramount pathological process in the context of Parkinson's disease. Intriguingly, some studies have suggested that nicotine may paradoxically elevate oxidative stress levels. This paradox warrants exploration, as it challenges our understanding of nicotine's positive impact on neurodegenerative diseases characterized by oxidative stress.

2. Materials and Methods

2.1. Animal Models of Parkinson's Disease

To facilitate effective in vivo studies on any disease, the selection of an animal model that faithfully recapitulates the pathogenesis of the actual ailment is of paramount importance. Parkinson's disease, in particular, has been the subject of various animal model investigations, with a range of approaches involving the induction of parkinsonism through neurotoxins such as 6-OHDA, rotenone, paraquat, and others [12]. Nevertheless, among these models, the one induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) stands out as the most reliable and robust choice. Its superiority stems from two key factors: firstly, the alterations it precipitates upon administration closely mirror the pathophysiological hallmarks of the actual disease; and secondly, it possesses the unique capability to induce parkinsonism in primates, including humans [13]. The pivotal mechanism underpinning the MPTP-induced model's effectiveness lies in its metabolic transformation by the enzyme MAO-B, resulting in the conversion of MPTP into its active form, MPP+. Subsequently, MPP+ avidly binds to the dopamine transporter (DAT) located on dopaminergic neurons within the substantia nigra. This binding event, in turn, triggers the demise of these neurons and disrupts mitochondrial function, mirroring the pathogenic cascade observed in Parkinson's disease [14].

The research was conducted on 160 albino laboratory mice, aged 8 weeks and weighing 22 ± 2g. The animals were housed, cared for, and tested by the "Guide for the Care and Use of Laboratory Animals" [15] at the Department of Biology, Iv. Javakhishvili Tbilisi State University, and its vivarium. Throughout the study, the mice had unrestricted access to both water and standard laboratory-grade food. The ambient room temperature was rigorously maintained at 22 ± 2 ℃ to ensure a stable and comfortable environment for the animals. Furthermore, the mice were subjected to a controlled 12-hour light-dark cycle to mimic their natural circadian rhythm. Before the commencement of any experimental procedures, the mice were allowed a period of 7 days for acclimatization to the vivarium conditions. Following this adaptation phase, they were systematically partitioned into four distinct experimental groups, each composed of 40 mice:

- Group G1 (control group): Mice received four intradermal injections of sterile physiological solution (0.9% NaCl) at 2-hour intervals, with a volume of 10ml/kg.
- Group G2: Mice were treated with drinking water containing nicotine bitartrate at a concentration of 200μg/ml, ad libitum for 14 days.
- Group G3: Mice received four intradermal injections of MPTP at 2-hour intervals, at a dose of 20mg/kg, with a volume of 10ml/kg.

- Group G4: Mice were injected intradermally with MPTP following the same protocol as Group G3, and 72 hours after the injection, they were given nicotine aqueous solution for 14 days, similar to Group G2.

The dosage of both the neurotoxin (MPTP Sigma-Aldrich) and nicotine was determined based on existing literature data [16,17]. A fresh nicotine solution was prepared every three days.

On the seventeenth day of the study, the animals from each group underwent behavioral tests: "Open field" and "The beam walking test". After the tests, animals were euthanized according to international guidelines (AVMA Guidelines for the Euthanasia of Animals: 2020 Edition). After euthanizing the animals, we decapitated them. Then their brains were removed and frozen at -80 ℃. Using a stereomicroscope (Laxco LMS-Z230PMZS33) and mouse brain atlas [18], we dissected the substantia nigra (Figure 1).

2.2. Behavioral Tests

We employed two distinct behavioral tests to evaluate the condition of the experimental animals, namely, the "Open field" test, which assesses both their motor and cognitive activity and the "The Beam walking test," designed to measure their coordination ability. These tests serve as valuable indicators of the presence of parkinsonism-like symptoms in the mice. The "Open field" test was conducted within a square-shaped enclosure measuring 90 x 90 x 70 cm, with the floor divided into 5 x 5 cm cells. The central portion of the enclosure, encompassing 16 cells, was designated as the center square. Each mouse was placed at the center, and their behavior was meticulously observed for a duration of 5 minutes. Several parameters were systematically recorded, including 1. the number of line crossings, 2. the frequency of entrance into the center square, 3. instances of rearing on the hind legs, and 4. the duration of immobility. The "Beam walking test" entailed the use of two wooden planks, each extending to a length of 1 meter, and suspended at a height of 80 centimeters above the ground. The upper plank gradually tapered from a width of 2.0 centimeters to 0.5 centimeters, with a consistent height of 1 cm, while the lower plank tapered from 4.0 to 2.5 centimeters in width. Positioned at the narrower end of the track was a designated "house." Mice were introduced at the wider end of the apparatus, and the test involved counting the instances when the mice's paws slipped from the wider end to the narrower end. Preceding the actual test, the mice underwent a comprehensive two-day "training" regimen to acclimate themselves to the apparatus and its demands.

2.3. Determination of Dopamine in the SN

We defined the content of dopamine in the Substantia nigra by ELISA Kit (MBS761192), according to the manufacturer's protocol.

2.4. Activities of the Antioxidant System

The technique used to assess superoxide dismutase (SOD) relies on the enzyme's ability to compete against tetrazole nitro blue in neutralizing superoxide anion radicals [19]. The procedure for measuring catalase (CAT) activity involved utilizing hydrogen peroxide's capacity to form a pigmented compound with molybdenum salts, and the intensity of the resulting color was assessed at a wavelength of 410 nm. To quantify the concentration of hydrogen peroxide (H_2O_2), we employed the assay kit provided by Mybiosource (MBS822356). The level of NO was determined by measuring the product ($NaNO_2$) generated from the reaction between NO and molecular oxygen (O_2) [20,21]. The concentration of malondialdehyde (MDA) was determined using the "General Lipid Peroxidation (MDA) Assay Kit" provided by Mybiosource (MBS822354).

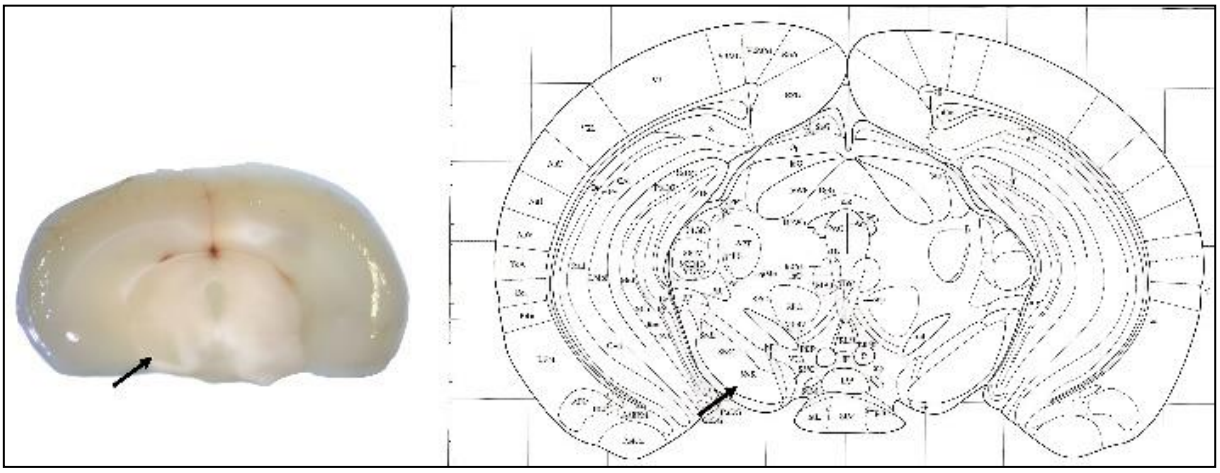


Figure 1. (A) Substantia nigra on the brain slice, and (B) according to the atlas (Paxinos et al. 2001)

2.5. Statistical Analysis

The data from each type of biochemical experiment were analyzed separately and treated by analysis of variance (ANOVA) with factor experimental condition experiments were repeated four times with triplicate samples for each experiment. When a significant effect was observed by ANOVA, Student's t-test was used to compare the samples.

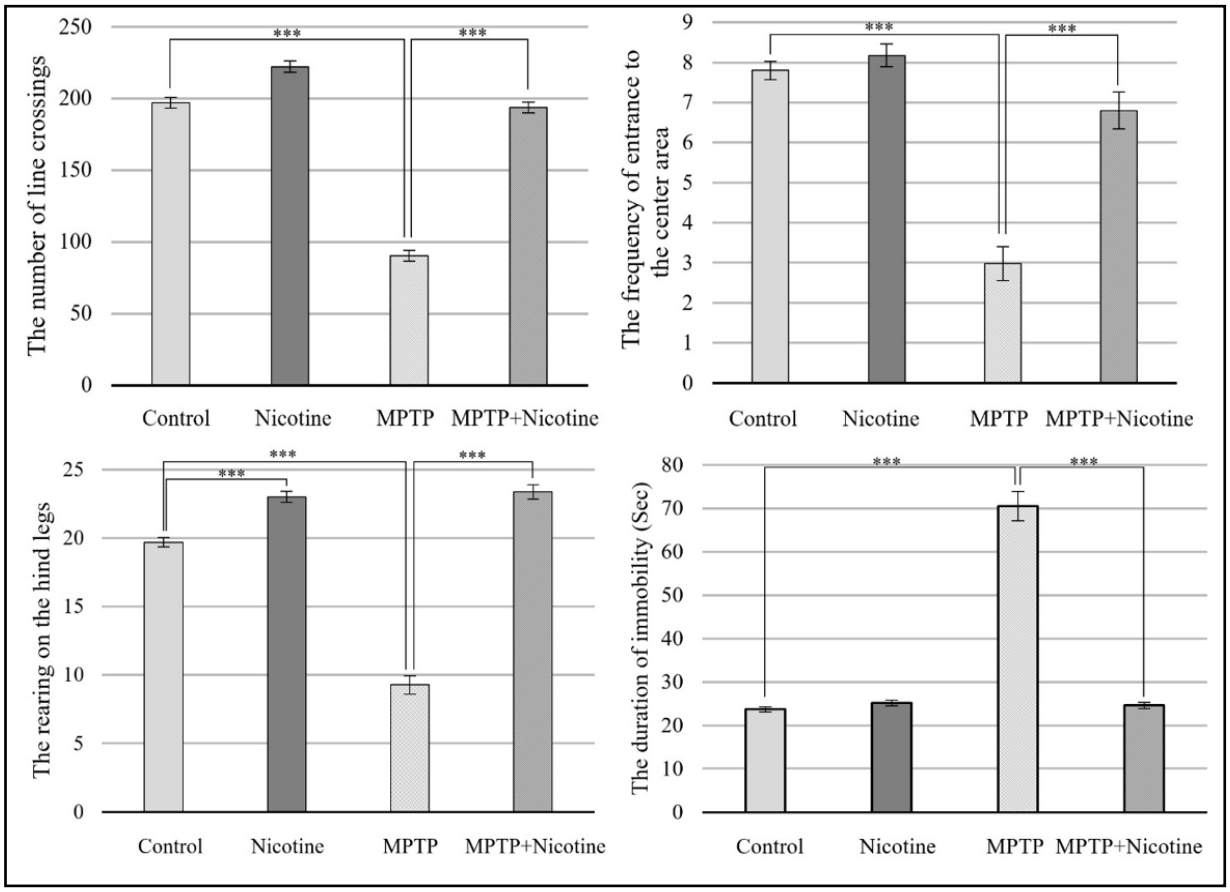
3. Results

3.1. Effects of Nicotine on the Psycho-Emotional State of Model Animals

As depicted in Figures 2A, B, and C, within the G3 group, there is a notable contrast when compared to the control group (G1). Specifically, we observe a significant reduction in the frequency with which the rodent stands on its hind legs in the field (approximately 60%), along with a similar decrease in the number of lines crossed (approximately 62%) and exits made in the center (≈60%). Remarkably, these impairments are effectively ameliorated following intraperitoneal nicotine administration.

Contrastingly, within the G3 group, the activity index shows a significant increase with the duration of contractions, as indicated in Figure 2D, marking a remarkable rise of approximately 200%. Nicotine intake similarly improves these data, approaching levels observed in the control group. Results of the G4 group consistently returned to normal or even outperformed the results of the G1 group.

The beam walking test yielded similar findings, with nicotine showing a remarkable 86.79% enhancement in the coordination of the experimental animals (Figure 3). In addition, the study emphasized that intact mice showed negligible alterations in response to nicotine.



Note: the data are mean ± SEM; The reported results represent the mean or average values obtained from 10 repeated trials. * P≤0.01; ** P≤0.001; *** P≤0.0001

Figure 2. Results of The Open Field Test. (A) The number of line crossings; (B) The frequency of entrance to the center area;(C) The rearing on the hind legs; (D) The duration of immobility (Sec).

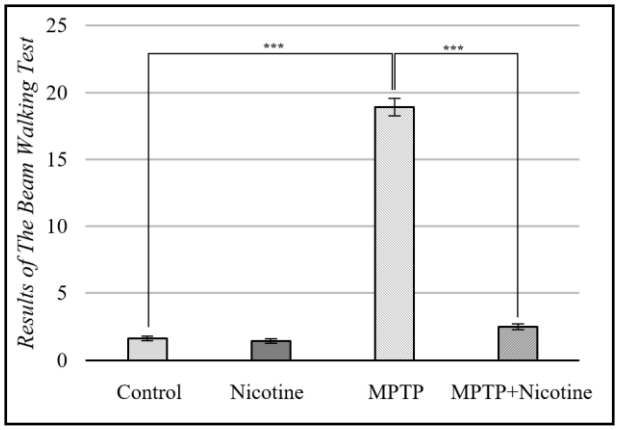


Figure 3. Results of The Beam Walking Test. The reported results represent the mean or average values obtained from 10 repeated trials. Note: Data are mean ± SEM; * P≤0.01; ** P≤0.001; *** P≤0.0001

3.2. The Impact of Nicotine on Dopamine Levels in Substantia Nigra Cells

Upon assessing behavioral parameters, we investigated how nicotine influenced dopamine levels in the substantia nigra cells across all four groups of experimental animals. Figure 4 illustrates that the dopamine quantity in the substantia nigra of mice within the G2 group markedly differs from that in the control animals (group G1). Conversely, dopamine levels in the G3 group animals are reduced by approximately 15% in comparison to the control group. Notably, nicotine consumption among mice treated with MPTP (G4 group) significantly elevated dopamine levels compared to the G3 group by approximately 23% (Figure 4)

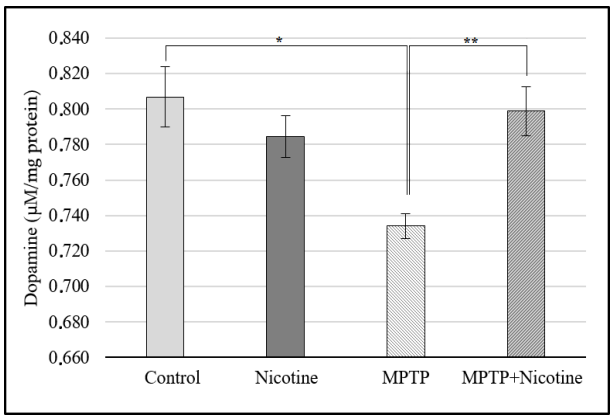


Figure 4. Nicotine's Impact on the Levels of Dopamine in the SN cells. The reported results represent the mean or average values obtained from 10 repeated trials. Note: Data are mean ± SEM;* P≤0.01;** P≤0.001; *** P≤0.0001

3.3. Influence of Nicotine on the Activity of Antioxidant system in Substantia Nigra Cells

In the subsequent phase of our research, it was illustrated that the neurotoxin significantly decreased levels of nitric oxide and malondialdehyde by approximately 75-85%, whereas nicotine reduced them by approximately 50% (Figure 5A and 5B). Regarding hydrogen peroxide, its levels increased in both the G3 and G2 groups. Nevertheless, in the G4 group, where animals were administered nicotine following MPTP injections, its concentration decreased by a notable 45.64% (Figure 5C).

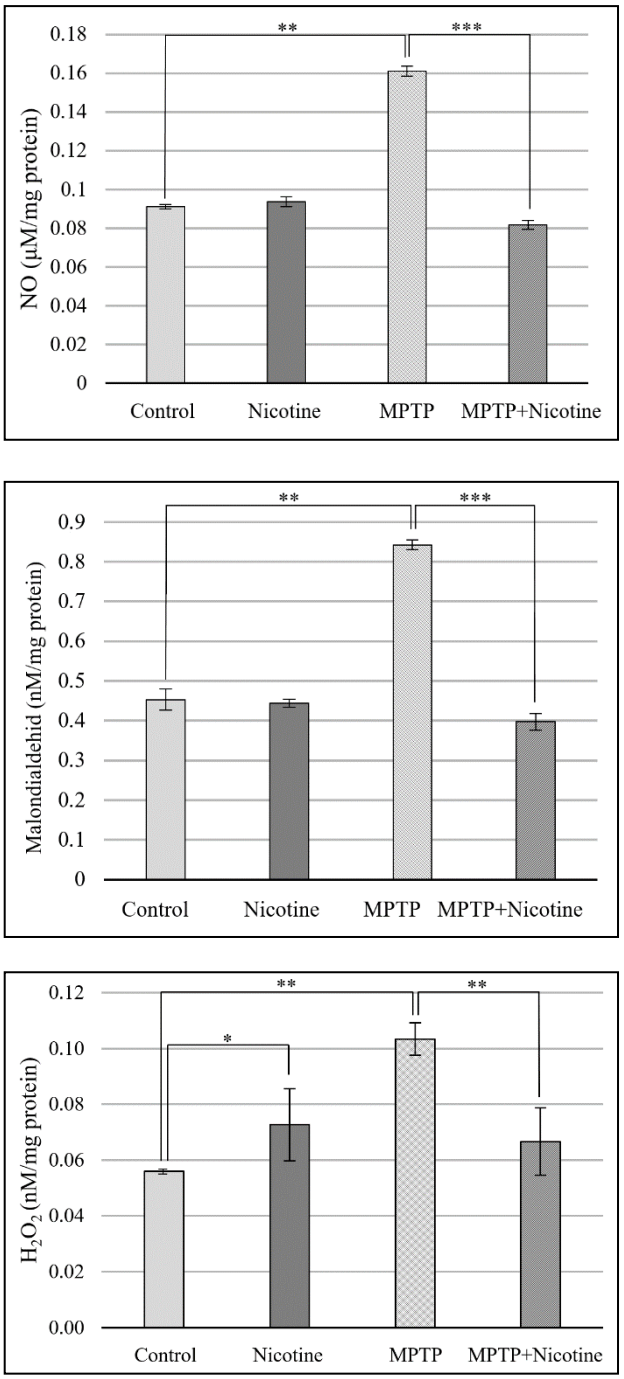


Figure 5. Nicotine's Impact on the Levels of (A) Nitric Oxide, (B) Malondialdehyde, and (C) Hydrogen Peroxide in SN cells. The reported results represent the mean or average values obtained from 10 repeated trials. Note: Data are mean ± SEM;* P≤0.01;** P≤0.001; *** P≤0.0001

As depicted in Figure 5, the results demonstrate a significant increase in the levels of active molecules,

including nitric oxide, hydrogen peroxide, and malondialdehyde, within the substantia nigra cells following MPTP treatment. This increase suggests the activation of peroxidative oxidative processes under these conditions. However, when nicotine was administered (G4), a substantial decrease in the levels of these molecules was observed. Considering these findings, our next objective was to investigate the extent of activation of antioxidant enzymes within the substantia nigra cells. To address this, we examined the presence of key enzymes such as superoxide dismutase and catalase in all four experimental groups. The results of this analysis are presented in Figure 6. An analysis of the enzymes of the antioxidant system revealed that the introduction of MPTP resulted in a decrease in the activity of superoxide dismutase by 12.39%. Conversely, during nicotine therapy, the activity of the enzyme increased by 35.18% (Figure 6 A). On the other hand, the introduction of nicotine and neurotoxin resulted in a decrease in catalase activity by 24.14% and 31.04%, respectively. However, in the G4 group, catalase levels increased (Figure 6 B).

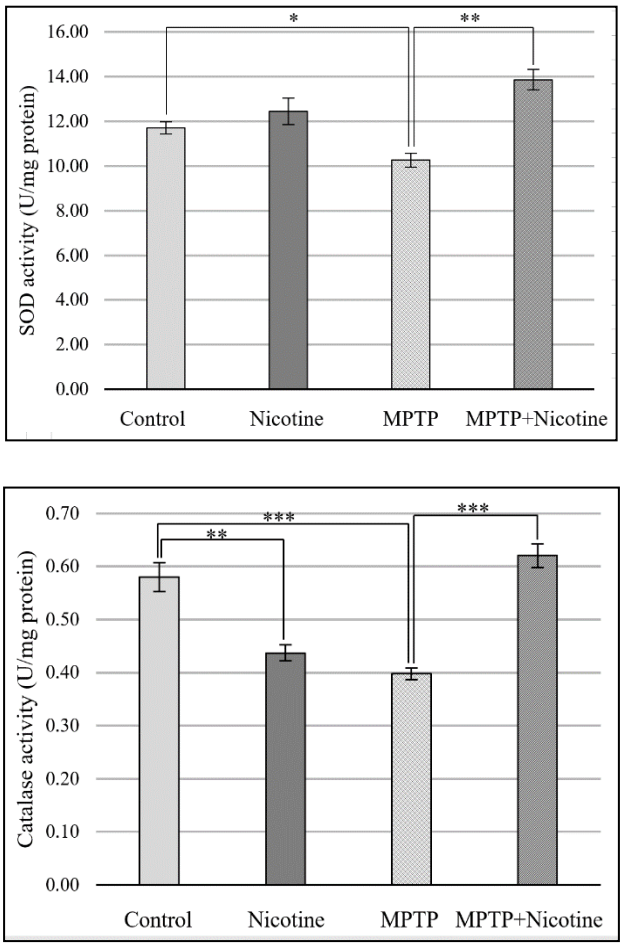


Figure 6. Effects of nicotine on antioxidant enzymes: (A) Superoxide dismutase and (B) Catalase activity in the SN cells. The reported results represent the mean or average values obtained from 10 repeated trials. Note: Data are mean ± SEM;* P≤0.01;** P≤0.001;*** P≤0.0001

4. Discussion

Parkinson's Disease (PD) is a progressive neurodegenerative disease characterized by the gradual loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of α-synuclein protein in Lewy bodies [22]. PD is believed to have multiple contributing factors, including aging, genetic predisposition, environmental factors that induce oxidative stress, mitochondrial dysfunction, and inflammatory processes. Various genetic mutations have also been explored, which can disrupt the ubiquitin-proteasome system and impact the organism's motor function. While levodopa is a primary treatment for PD, alternative methods and therapeutic agents for addressing the disease's pathogenesis are continuously sought after.

Notably, epidemiological studies have suggested that tobacco use may reduce the risk of developing PD. In experimental studies, nicotine has been shown to decrease the levels of SIRT6 in neuronal cultures and the brain, leading to speculation that the neuroprotective effects of nicotine may be attributed to this property [19]. Oxidative stress is considered a key pathological mechanism in PD development, prompting research into various antioxidant compounds as potential therapeutic options. This category includes compounds like coenzyme Q, glutathione, vitamins C and E, flavonoids, and polyphenols[5], with nicotine also being considered as a potential therapeutic compound [23] However, the extent of nicotine's antioxidant properties remains relatively understudied. For instance, while nicotine is known to increase catalase activity in PD, there is limited evidence regarding its impact on other enzymes within the antioxidant system [24]. Considering these factors, the primary objective of our experiment was to investigate the impact of nicotine on the antioxidant system within Substantia nigra cells under conditions associated with Parkinson's Disease (PD). It is well-established that oxidative stress plays a pivotal role in the development of PD, stemming from the accumulation of reactive oxygen species (ROS) or their improper elimination, resulting in damage to lipids, proteins, DNA, and ultimately, cell death. Numerous studies have indicated the activation of this process in PD [25]. Intriguingly, nicotine administration under these conditions reliably amplifies the presence of these molecules within the substantia nigra pars compacta cells. Additionally, MPTP treatment leads to an elevation in malondialdehyde levels within the substantia nigra cells, signifying the activation of oxidative processes within the brain cells of affected individuals. However, it is noteworthy that mice in group 4 exhibit a significant decrease in malondialdehyde levels under these conditions, as depicted in Figure 5B. Importantly, these findings are consistent with existing literature. Furthermore, the exogenous administration of nicotine into the substantia nigra pars compacta cells of experimental animals resulted in increased activity of antioxidant enzymes, namely

superoxide dismutase and catalase. While some studies suggest that nicotine may exacerbate oxidative stress, it is worth noting that this stands in contrast to the observed positive effects of nicotine on neurodegenerative diseases associated with oxidative stress. The study examining the impact of nicotine on the antioxidant system in a Parkinson's disease model demonstrated an increase in the activity levels of superoxide dismutase and catalase, leading to a reduction in malondialdehyde and hydrogen peroxide levels. Additionally, the concentration of nitric oxide decreased. It is noteworthy that the effects of nicotine are prominent solely in the Parkinson's disease model and do not affect healthy animals. In some cases, nicotine may even hurt catalase and hydrogen peroxide. This finding partly explains why certain studies have reported a correlation between nicotine and increased oxidative stress [26]. It is noteworthy that the impact of nicotine on dopamine levels was also evident in the substantia nigra cells. The quantity of this hormone in the control (G1) and G2 groups, which exclusively received nicotine throughout the experiment, showed minimal variation and lacked significant distinctions. However, notable disparities emerged in the case of animal models afflicted with Parkinson's disease (group G3) and those in group G4. In the latter group, dopamine levels exhibited an approximate 30% increase in comparison to group G3. However, the reason why the beneficial properties of nicotine are observed exclusively in Parkinson's disease not in the control animals remains unclear and requires detailed research.

5. Conclusions

Since the MPTP model is considered a valid model of Parkinson's disease, our data indirectly indicate the possible antioxidant activity of nicotine, which may be a useful observation for the development of additional means of combating this pathology. In summary, this study provides compelling evidence of nicotine's potential neuroprotective effects in PD. Nicotine improved motor deficits, enhanced coordination, and exhibited antioxidative properties, reducing oxidative stress markers and bolstering antioxidant enzyme activity. These findings hold promise for further research and the development of targeted therapies leveraging nicotine's benefits while ensuring safety and efficacy in PD treatment. Nonetheless, rigorous clinical investigations and continued exploration of nicotine's mechanisms are warranted for its potential application in PD therapy. It is also necessary to thoroughly study the side effects of nicotine and weigh all the risks.

Ethics Approval

Research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU).

Conflict of Interest

The authors report no conflict of interest.

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