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The Effects of Vortioxetine on Rotenone-Induced Inflammatory Changes in Rat-Derived Enteroglial Cells: The Role of the TLR4/NFκB Signaling Pathway

Sıçan Kökenli Enteroglial Hücrelerde Rotenon ile İndüklenen İnflamatuvar Değişiklikler Üzerine Vortioksetinin Etkileri: TLR4/NFκB Sinyal Yolağının Rolü

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Abstract: Parkinson's disease (PD) is a progressive neurodegenerative disorder with both motor and non-motor symptoms, and currently, there is currently no disease-modifying therapy. Due to their potential anti-inflammatory effects, antidepressants have gained attention as therapeutic agents in inflammation-related neurological conditions. In this study, we aimed to investigate the effects of vortioxetine on rotenone-induced enteric inflammation in an *in vitro* model using enteric glial cells and whether these effects involve modulation of the TLR4/NF-κB signaling pathway. Cells were treated with rotenone (10 μM) and vortioxetine (1 and 5 μM). TLR4 and NF-κB mRNA expression levels were analyzed by RT-qPCR, and the levels of TNF-α, IL-1β, and IL-6 were measured via ELISA. The findings showed that rotenone significantly suppressed TLR4 and NF-κB expression by impairing the immune responses of glial cells, and the administration of 5 μM vortioxetine further enhanced this effect. Additionally, the decrease observed in TNF-α and IL-1β levels in the rotenone groups was reversed by vortioxetine administration. The results suggest that vortioxetine may regulate inflammatory responses in enteric glial cells through the TLR4/NF-κB pathways and could be investigated as a potential therapeutic compound in inflammation-based models of the gut-brain axis in PD.
Keywords: Enteric inflammation, Enteric glia, Rotenone, Toll-like receptor, Vortioxetine

Özet: Parkinson hastalığı (PH) progresif bir nörodejeneratif hastalık olup günümüzde hastalığı durdurmaya yönelik kesin bir tedavi seçeneği bulunmamaktadır. Gastrointestinal inflamasyon, PH ile ilişkili motor-olmayan bulgulardan biridir. Son yıllarda antidepresanların potansiyel antiinflamatuvar etkileri nedeniyle nörodejeneratif hastalıkların tedavisinde kullanılabileceğine dair ilgi artmıştır. Bu çalışmada, vortioksetinin enterik glia hücrelerinde rotenon ile indüklenen inflamatuvar yanıtlar üzerindeki etkisi ve bu etkisinde TLR4/NF-κB sinyal yolağının rolü araştırılmıştır. Rotenon (10 μM) ve vortioksetin (1 ve 5 μM) ile muamele edilmiş hücre örneklerinde TLR4 ve NF-κB mRNA ekspresyonları RT-qPCR ile, TNF-α, IL-1β ve IL-6 düzeyleri ise ELISA yöntemiyle değerlendirilmiştir. Bulgular, rotenonun glial hücrelerin immün yanıtlarını bozarak TLR4 ve NF-κB ekspresyonunu belirgin şekilde baskıladığını ve bu etkinin 5 μM vortioksetin uygulamasıyla daha da arttığını göstermiştir. Ayrıca rotenon gruplarında TNF-α ve IL-1β düzeylerinde gözlenen düşüş, vortioksetin uygulaması ile tersine dönmüştür. Sonuçlar, vortioksetinin enterik glia hücrelerinde TLR4/NF-κB yolakları üzerinden inflamatuvar yanıtı düzenleyebileceğini ve PH'nin bağırsak-beyin eksenine dayalı inflamasyon modelinde potansiyel bir terapötik madde olarak çalışılabileceğini göstermektedir.
Anahtar Kelimeler: Enterik inflamasyon, Enterik glia, Rotenon, Toll-benzeri reseptör, Vortioksetin.

Ethics Committee Approval: This study was conducted using a commercially available cell line. No human participants, human-derived tissues, or animal models were involved. Therefore, ethical approval was not required for the experimental procedures described. We confirm that all methods were carried out in accordance with relevant guidelines and regulations.
Informed Consent: The authors declared that it was not considered necessary to get consent from the patients because the study was a retrospective data analysis.
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1. Introduction

Post-mortem studies have shown that Lewy body pathology, a characteristic feature of Parkinson's disease (PD), exists in the enteric nervous system (ENS) beyond the central nervous system (CNS) in nearly all PD patients (1). The ENS referred to as the "second brain" functions as the intrinsic neural network of the gastrointestinal tract and exhibits numerous similarities to the CNS (2). The ENS has a very large number of neurons and a larger population of glial cells (3). Enteric glial cells (EGCs) closely resemble CNS astrocytes and microglia in both structure and function (4). During inflammation, EGCs activate signaling pathways that result in the release of cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 (5).

Gastrointestinal dysfunction, which belongs to the non-motor symptoms of PD, is believed to emerge years before the onset of motor symptoms (6). With the identification of Lewy body pathology in enteric neurons obtained from biopsies of PD patients, the potential involvement of the ENS in PD pathophysiology has gained attention (7). Studies have shown that EGCs actively regulate the neuroimmune axis via pattern recognition receptors such as Toll-like receptors (TLRs) (8). TLR4 plays a crucial role in the pathogenesis of PD by mediating neuroinflammation, responding to alpha-synuclein aggregates, and contributing to both CNS and ENS dysfunction, making it a promising therapeutic target (9, 10).

Pharmacological approaches have been used to develop experimental models that mimic nigrostriatal neurodegeneration and PD-like pathology in animals (2). Exposure to rotenone, a pesticide, is widely utilized both *in vitro* and *in vivo* as a disease model because it replicates key pathological and behavioral features of PD (11, 12). Rotenone administration has been shown to induce pathological alterations not only in the CNS but also in the ENS, leading to both motor and non-motor symptoms, including gastrointestinal dysfunction (6, 13). Rotenone is a highly lipophilic molecule and a well-characterized inhibitor of mitochondrial complex I. In addition to inducing dopaminergic neurodegeneration, rotenone exposure activates inflammatory pathways through mechanisms such as p38 MAPK activation and mTOR inhibition, leading to increased oxidative stress, ATP depletion, and apoptosis (14). Furthermore, rotenone exposure has been shown to upregulate pro-inflammatory cytokines and TLR-related signaling in both neuronal and glial cells (15). In enteric neuronal cell cultures, rotenone exposure has been shown to increase the number of α-synuclein inclusions within non-neuronal (16). Moreover, following rotenone exposure, TLR4 knockout mice displayed reduced intestinal inflammation, gut and motor dysfunction, neuroinflammation, and neurodegeneration compared to wild-type mice (17), indicating that PD symptomatology could be related to disrupted immune responses mediated by EGCs (8).

Experimental studies have shown that certain antidepressants can reduce proinflammatory cytokine levels in inflammatory conditions (18). Vortioxetine is an antidepressant that works in multiple ways by blocking 5-HT_3, 5-HT_7, and 5-HT_{1D} receptors with 5-HT_{1B} partial agonism and 5-HT_{1A} agonism. It also inhibits the serotonin transporter (SERT) (19). Previous research has reported that vortioxetine may exert beneficial effects on TLR2-mediated inflammatory mechanisms (20, 21). Identifying strategies that can prevent enteric neurodegeneration may contribute to the development of new therapeutic principles for neurodegenerative diseases. This study aims to investigate the inhibitory effects of vortioxetine on rotenone-induced inflammatory changes in EGCs through modulation of the TLR4/NFκB signaling pathway.

2. Materials and Methods

2.1. *In vitro* studies

2.1.1. Chemicals and reagents

Vortioxetine was sourced by H. Lundbeck A/S (Denmark). For experimental treatments, rotenone and the solvent dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vortioxetine was prepared in 10% 2-hydroxypropyl-β-cyclodextrin, while rotenone was dissolved in DMSO. The final concentration of DMSO in the culture medium was kept below 0.2% to prevent cytotoxicity.

2.1.2. Enteroglial cell culture

The EGC cell line used in this study, derived from rat tissue, was generously obtained from Dr. Luca Antonioli (University of Pisa). Cells were cultured in DMEM enriched with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. When cells

reached 70–80% confluency, they were detached using trypsin-EDTA and subsequently seeded into T-25 flasks or 96-well plates for experimental procedures.

2.1.3. Study design

The selection of rotenone and vortioxetine concentrations was based on our previous study, where we optimized these concentrations through viability and functional assays (21). The experimental groups established for the study are detailed in Table 1.

Table 1. Experimental groups

Group	Treatment
I. CONTROL	Culture medium without any additives
II. ROTENON	Culture medium containing 10 μM rotenone
III. VORTIOXETINE (V1)	Culture medium containing 1 μM vortioxetine
IV. VORTIOXETINE (V2)	Culture medium containing 5 μM vortioxetine
V. ROT+V1	Culture medium containing 10 μM rotenone and 1 μM vortioxetine
VI. ROT+V2	Culture medium containing 10 μM rotenone and 5 μM vortioxetine

2.1.4. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cultured cells using a commercial RNA extraction kit (HibriGen, Cat. No. MG-RNA-01-100) in accordance with the supplier's guidelines. After cell lysis, RNA was collected using spin column-based purification. RNA quality and quantity were evaluated using spectrophotometric analysis, and only samples with a 260/280 absorbance ratio between 1.7 and 2.0 were selected for subsequent steps. For cDNA synthesis, 1 μg of RNA was converted to cDNA using the OneScript® Plus cDNA synthesis kit (ABM, G236). Reverse transcription was performed at 25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min. cDNA was stored at −80 °C until further use.

Specific primers for TLR4 and NFκB that are used in the study are listed in Table 2. PCR amplification was performed using a Roche LightCycler 96 system. Cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. A melting curve analysis (60 °C to 95 °C, with 0.5 °C/s increments) confirmed specificity. Gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method (22) using the reference gene beta-actin (β-act) for normalization.

Table 2. Primer sequences used for RT-qPCR

Gene	Primer Sequence
TLR4	F: GGATGATGCCTCTCTTGCAT
	R: TGATCCATGCATTGGTAGGTAA
NFκB	F: GCCTGACACCAGCATTTGA
	R: CAAACCAAACAGCCTCACG
β-actin	F: CGGCAATGAGCGGTTCC
	R: TGCCACAGGATTCCATACCC

2.1.5. Measurements of TNF-α, IL-1β, and IL-6 levels

Levels of TNF-α, IL-1β, and IL-6 in EGC lysates were measured using enzyme-linked immunosorbent assay (ELISA) kits (BT Lab; TNF-α: E0764Ra, IL-1β: E0119Ra, IL-6: E0135Ra), following the manufacturers' protocols. Briefly, cells were washed with PBS, detached with trypsin, and collected by centrifugation at 1000 g for 5 minutes. After discarding the supernatant, cells were washed three times with PBS. A total of 1×10^6 cells were resuspended in PBS and subjected to three freeze-thaw cycles. Lysates were centrifuged at 1500 g for 10 minutes at 2–8 °C, and the resulting supernatants were collected and stored at −20 °C until analysis. All measurements were performed in duplicate.

2.3. Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). The Kolmogorov–Smirnov test was used to assess the normality of distribution. Group differences were assessed with one-way

ANOVA followed by Tukey's post-hoc test. For non-normally distributed data, the Kruskal–Wallis test was applied, followed by Dunn's post-hoc test for multiple comparisons. Statistical analyses were performed on GraphPad Prism 10.0 software (SanDiego, CA, USA), and a p-value < 0.05 was considered statistically significant.

3. Results

3.1 Changes in TLR4 and NFκB mRNA expression

The mRNA expression levels of TLR4 and NFκB were assessed in EGCs to compare differences among experimental groups. As shown in Figure 1a, Rotenone exposure caused a marked decrease in TLR4 expression ($p < 0.0001$), indicating a toxic impairment of inflammatory signaling in EGCs. Co-administration of vortioxetine (5 µM) with rotenone further decreased TLR4 expression ($p < 0.05$ vs. rotenone alone), suggesting that vortioxetine potentiated the rotenone-induced TLR4 downregulation rather than reversing it. Interestingly, vortioxetine administered alone at both concentrations (1 µM and 5 µM) also significantly reduced TLR4 expression compared to the control group ($p < 0.0001$ for both), indicating that vortioxetine may directly modulate basal TLR4 signaling even in the absence of rotenone Unlike TLR4, NF-κB expression did not show significant differences between groups (Figure 1b). However, in the rotenone-treated group, NF-κB expression was reduced by 48.83%, indicating a marked toxic effect. When high-concentration vortioxetine (5 µM) was co-administered with rotenone, NF-κB levels decreased even further (52.67% reduction), suggesting that vortioxetine at this concentration did not mitigate, and may even potentiate, rotenone-induced suppression. Interestingly, the combination of rotenone with a lower concentration of vortioxetine (1 µM) resulted in a slight 18.61% increase in NF-κB expression compared to rotenone alone; however, this change did not reach statistical significance ($p > 0.05$).

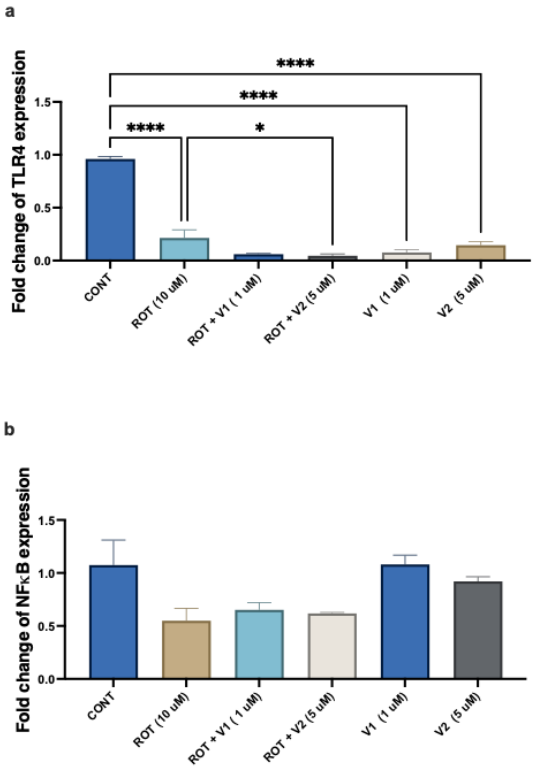

Figure 1. Fold changes in TLR4 and NF-κB mRNA levels in EGCs. Data are presented as mean ± SEM. Experiments were performed in duplicate and repeated three times. Statistically significant results are marked with an asterisk (*). CONT: Control, ROT: 10 µM Rotenone, V1: 1 µM vortioxetine. V2: 5 µM vortioxetine. *$p < 0.05$, ****$p < 0.0001$.

3.2. Changes in proinflammatory cytokines levels in EGCs

TNF-α levels were significantly reduced in the rotenone-treated group compared to the control ($p < 0.05$), indicating a suppressive effect of rotenone alone (Figure 2a). When vortioxetine was co-administered with rotenone, both low (1 µM) and high (5 µM) concentrations, did not change the TNF-α levels. Moreover, vortioxetine alone (at both concentrations) did not significantly alter TNF-α levels compared to the control group.

For IL-1β levels, although there was a 32% reduction following rotenone administration compared to the control group, which may be considered physiologically relevant, the difference did not reach statistical significance (Figure 2b). Co-treatment with vortioxetine at 1 µM significantly increased IL-1β levels compared to rotenone alone ($p < 0.01$), while 5 µM vortioxetine showed non-significant reversal. Vortioxetine alone (at both concentrations) did not significantly alter IL-1β levels compared to the control group.

IL-6 levels did not differ significantly between the treatment groups, indicating that neither rotenone nor its combination with vortioxetine led to a significant modulation of IL-6 expression (Figure 2c).

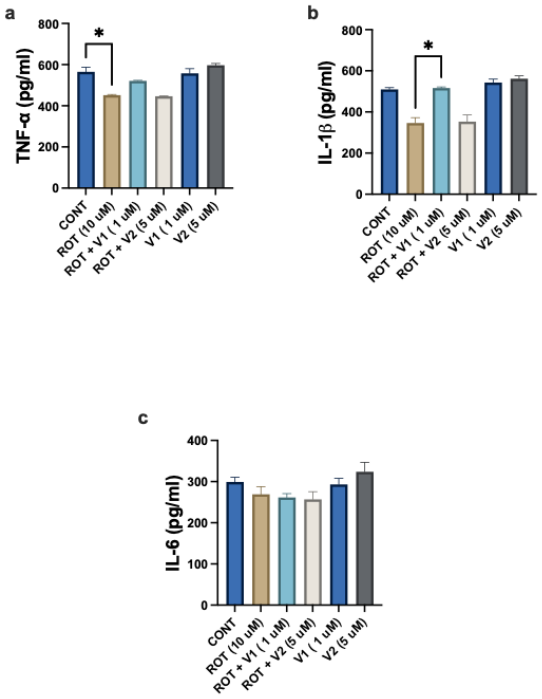

Figure 2. Changes in TNF-α (a), IL-1β (b) and IL-6 (c) levels in enteric glial cells. Data are presented as mean ± SEM. Experiments were performed in duplicate and repeated three times. Statistically significant results are marked with an asterisk (*). CONT: Control, ROT: 10 µM Rotenone, V1: 1 µM vortioxetine. V2: 5 µM vortioxetine. *$p < 0.05$.

4. Discussion

PD pathology is estimated to begin in the ENS and transfer to the CNS via vagal nerve (23) supported by the research indicating pathological α-synuclein aggregates detected in gastrointestinal tissues several years prior to clinical diagnosis of PD (24). Given the importance of ENS in the disease progression, our study provides valuable insight into the potential neuroprotective role of vortioxetine in rotenone-induced EGC dysfunction. By examining both gene expression and inflammatory cytokine levels, our findings demonstrated that rotenone, a complex I inhibitor, disrupted the cellular inflammatory response and led to a reduction in inflammatory marker levels. On the other hand, vortioxetine appeared to enhance this response, as indicated by an increase in the expression of these markers. These findings highlight vortioxetine's potential in modulating early enteric inflammatory pathways relevant to PD pathogenesis.

Rotenone is a pesticide that inhibits the complex I of the mitochondrial electron transport chain. It is widely used in research to model PD because it can replicate many of the disease's key features (25). Exposing the stomach to the rotenone caused the propagation of α-synuclein to the brain (26, 27). Rotenone has also been shown to induce pathological changes in enteric neuronal culture (16, 28) and primary enteric neurons (29). In our previous study, we also demonstrated the toxic

effects of rotenone on EGCs (21). We performed this study in EGCs due to their essential roles in the gut inflammation (30). These cells functionally and morphologically resemble astrocytes in the CNS and are activated during inflammation through TLRs (31). Especially, the TLR4/NF-κB pathway plays a significant role in the pathogenesis of PD by mediating neuroinflammation and contributing to neuron damage (32). The TLR4/NF-κB pathway is activated in response to α-synuclein and other inflammatory stimuli, leading to increased expression of inflammatory genes and cytokines such as TNF-α and IL-1β (33, 34). Perez-Pardo et al. demonstrated that patients with PD exhibit increased intestinal TLR4 expression, mucosal immune activation, and disrupted gut barrier integrity, all of which were replicated in rotenone-treated wild-type mice (17). Our results showed that rotenone reduced TLR4 and NFκB levels in EGCs, suggesting that exposure to neurotoxins like rotenone may impair glial responses to inflammation by disrupting mitochondrial function. Consistent with our study, Rabaneda-Lombarte et al. demonstrated that rotenone impairs glial immune responses by disrupting cellular metabolism and interfering the metabolic reprogramming essential for glial activation (35, 36). The reduction of TLR4 expression observed with vortioxetine alone, as well as the further reduction seen with its co-administration alongside rotenone, might be explained by vortioxetine's intrinsic serotonergic immunomodulatory effects. In fact, chronic antidepressant treatment (including SSRIs) has been shown to attenuate TLR4 levels (37). This potentiation may also reflect a compensatory mechanism to prevent an exaggerated inflammatory response under toxic conditions in which cells can develop tolerance by reducing TLR4 receptor levels or responsiveness (38). In addition to its known central effects, recent clinical evidence suggests that vortioxetine may also exert therapeutic actions by modulating the gut microbiota composition (39). These findings raise the possibility that vortioxetine's immunomodulatory effects against gut-related inflammatory responses.

Proinflammatory cytokines are known to be increased in PD patients in serum (40), cerebrospinal fluid (41), brain (42) and even colon tissue (43). However, *in vitro* systems may fail to mimic these inflammatory responses because they lack the intricate cellular interactions present *in vivo*. Previous studies have demonstrated that rotenone-induced neurotoxicity is significantly amplified in the presence of glial cells, particularly microglia, through mechanisms involving oxidative stress and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (44). Given the functional parallels between EGCs and CNS glia, it is plausible that rotenone may also activate inflammatory signaling and oxidative responses in EGCs, thereby exacerbating cellular damage. This could explain why we observed a significant decrease in the levels of TNF-α and IL-1β in our model. Administration of a low concentration of vortioxetine increased the IL-1β levels in rotenone group, suggesting that it helps to regain cells to produce a response against inflammation. These findings highlight the relevance of glial cell type in shaping cellular vulnerability to mitochondrial toxins and emphasize the importance of considering glial contributions when modeling gut-related aspects of PD.

This study has some limitations. The findings are based on a single *in vitro* model using rat-derived EGCs, which, although highly relevant to the enteric nervous system, do not fully replicate the complex multicellular and microenvironmental interactions observed *in vivo*. Moreover, the glial response to rotenone and vortioxetine was assessed at the mRNA and cytokine levels; however, additional analyses would provide a more comprehensive understanding of the underlying mechanisms.

In conclusion, our findings provide novel insights into the effects of rotenone and vortioxetine on inflammatory responses in EGCs, highlighting the involvement of the TLR4/NF-κB pathway. Rotenone-induced suppression of inflammatory signaling suggests that mitochondrial toxins impair glial capacity to respond to environmental stressors. Importantly, co-treatment with vortioxetine, particularly at low concentrations, partially restored TLR4 expression and increased IL-1β levels, suggesting a potential modulatory role on glial-driven inflammation. These results support the hypothesis that targeting enteric glia and gut inflammation may offer promising avenues for early intervention in PD.

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