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Hydroxytyrosol Attenuates Hippocampal Inflammation and Damage in an Experimental Model ofParkinson's Disease: The Critical Role of the JNK–c-Jun–AP-1 Signaling Pathway in Neuroprotection

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Running title

Hydroxytyrosol Mitigates Hippocampal Inflammation in PD

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder marked by the loss of dopaminergic neurons in the substantia nigra, resulting in motor and non-motor symptoms. Inflammation caused by microglia in the hippocampus and overactivation of the JNK–c-Jun–AP-1 signaling pathway are key mechanisms involved in cognitive deficits associated with PD. This study examined the anti-inflammatory, neuroprotective, and antioxidant properties of hydroxytyrosol (HT) in a rat model of Parkinson's disease caused by reserpine (RES). Male Wistar rats were divided into five groups: control, vehicle + saline (VR + NS), RES + NS, HT + VR, and RES + HT. Behavioral assessments, including catalepsy, rotarod, beam walking, Y-maze, and passive avoidance tests, were conducted to evaluate motor and cognitive functions. Additionally, oxidative stress biomarkers, pro-inflammatory cytokines, acetylcholinesterase (AChE) activity, gene expression of the JNK/c-Jun/AP-1 pathway, and the density of dark neurons in the hippocampus were measured. HT administration significantly enhanced motor and cognitive performance, lowered oxidative stress markers, regulated cytokine levels, inhibited JNK–c-Jun–AP-1 activation, and reduced hippocampal dark neuron density. These findings highlight HT's neuroprotective potential against RES-induced neuronal injury, suggesting its promise as an additional therapeutic approach to mitigate inflammation and preserve cognitive function in PD.

Keywords: Parkinson's disease; hippocampus; inflammation; hydroxytyrosol; cytokines

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting more than one percent of the elderly population worldwide [1]. It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra, leading to key motor symptoms such as resting tremor, bradykinesia, muscle rigidity, and postural instability. In addition to motor problems, patients often face non-motor issues like depression, anxiety, sleep disturbances, pain, attention and memory problems, cognitive decline, and autonomic dysfunction, all of which significantly reduce quality of life [2].

The hippocampus, a key region for learning and memory, plays a crucial role in the development of cognitive impairments in PD. Microglial-driven inflammation in this region is closely associated with abnormal protein aggregation, including α-synuclein and tau, which contribute to synaptic damage and neuronal dysfunction [3]. Thus, hippocampal inflammation represents both a biomarker of disease progression and a promising therapeutic target for anti-inflammatory interventions.

Among the molecular pathways implicated in PD pathogenesis, the JNK–c-Jun–AP-1 signaling cascade is significant. Activated in response to cellular stress, this pathway phosphorylates c-Jun, forming the AP-1 complex that regulates transcription of pro-inflammatory and pro-apoptotic genes [4]. In PD, hyperactivation of JNK–c-Jun–AP-1 contributes to neuronal death in the substantia nigra and hippocampus, elevated inflammatory cytokine levels, exacerbated oxidative stress, and abnormal protein phosphorylation. These processes amplify microglial activation and synaptic injury, thereby worsening cognitive deficits. Consequently, inhibiting this pathway may represent an effective strategy for reducing neuroinflammation and preserving cognitive function [5].

To investigate PD mechanisms, several animal models have been developed. Reserpine (RES), a vesicular monoamine transporter 2 (VMAT2) inhibitor, depletes dopamine stores and induces motor and non-motor symptoms resembling PD. This model is associated with α-synuclein accumulation, impaired autophagy, and reduced tyrosine hydroxylase expression in the substantia nigra. Due to its simplicity and reproducibility, the RES model is widely utilized to investigate disease mechanisms and assess potential anti-PD agents [6].

Current pharmacological treatments for PD primarily alleviate symptoms but fail to halt neurodegeneration. The long-term efficacy of dopaminergic drugs diminishes, often leading to motor fluctuations and dyskinesia, while non-motor symptoms typically remain refractory. Therefore, novel therapeutic approaches capable of improving both motor and non-motor manifestations are urgently needed [7].

Plant-derived antioxidants have attracted considerable attention due to their ability to scavenge free radicals and mitigate oxidative stress. Hydroxytyrosol (HT), the principal polyphenol in extra virgin olive oil, exhibits potent

antioxidant and anti-inflammatory properties that confer neuroprotection [8]. HT suppresses NF-κB signaling, reduces pro-inflammatory cytokines such as TNF-α and IL-1β, and enhances anti-inflammatory mediators including IL-10 and TGF-β, thereby supporting neural homeostasis. Experimental studies have demonstrated that HT downregulates hippocampal inflammatory cytokines and exerts significant protective effects [9].

Mechanistically, HT reduces reactive oxygen species generation and inhibits JNK activation, c-Jun phosphorylation, and the formation of the AP-1 complex. This suppression limits the transcription of pro-inflammatory and pro-apoptotic genes, thereby shifting the signaling balance toward cell survival and repair [10]. As a result, neuronal function in the substantia nigra and hippocampus is preserved, and neurodegeneration is attenuated. Thus, inhibition of the JNK–c-Jun–AP-1 pathway constitutes a central mechanism underlying the neuroprotective effects of HT in PD models [11].

This study aims to elucidate the anti-inflammatory and neuroprotective effects of HT and their relationship to the JNK–c-Jun–AP-1 pathway in the hippocampus of RES-induced PD rats.

Materials and Methods

Animals

This study involved 60 male Wistar rats, around 8 months old and weighing 200 ± 20 g. They were kept in groups of five within transparent plastic cages measuring 16 × 37 × 30 cm. The environment was controlled at 22 ± 1 °C with 45 ± 5% humidity and a 12-hour light/dark cycle (lights on at 6:00 a.m.). Rats had free access to standard chow and water. To acclimate and monitor food intake, they were housed for one week at the Islamic Azad University, Shiraz Branch, without any intervention. All procedures were approved by the Animal Ethics Committee (Approval Code: IR.IAU.SHIRAZ.REC.1404.266), with efforts to minimize pain and discomfort. The number of animals was kept to the minimum essential for the study.

Drugs

HT, with a purity of ≥ 98%, and RES were obtained from Sigma-Aldrich (St. Louis, MO, USA). HT was dissolved in normal saline (NS) and given at a dose of 20 mg/kg body weight [12] (Ansari et al., 2025). RES was first dissolved in 0.3 mL of glacial acetic acid and then diluted with distilled water to create a solution with a concentration of 0.2 mg/mL. In the vehicle group (VR), glacial acetic acid and distilled water served as controls [13]. All solutions were freshly prepared daily and stored at 4 °C between doses.

Experimental Design

Rats were randomly divided into five groups (n = 12 each): a control group, a VR + NS group, a RES + NS group, an HT + VR group, and a RES + HT group. RES was administered intraperitoneally at a dose of 0.2 mg/kg daily for 13 days. Once Parkinsonian-like motor symptoms were confirmed, HT was administered orally via gavage at 20 mg/kg for 14 days [14].

Motor Tests

Catalepsy assessment: At the end of HT treatment, catalepsy was evaluated in all groups (n = 12 per group) using the bar test. The apparatus consisted of a horizontal wooden bar (0.9 cm diameter) positioned 9 cm above the platform. Each rat's forepaws were placed on the bar, and the time the animal maintained this posture was recorded. The maximum cutoff time was 30 s. The test ended when at least one forepaw was removed or when active exploratory head movement occurred. Each rat was tested three times with 3 min intervals [13].

Rotarod performance: Motor coordination and balance were evaluated using an accelerating rotarod device (Borj Sanat, Iran). Rats (n = 12 per group) were placed on a rotating rod, and latency to fall was recorded. Training sessions (3 sessions, each with 3 trials at 10-minute intervals) were conducted prior to RES administration to ensure equal

learning. The rod accelerated from 4 to 40 rpm over a period of 5 minutes. Experimental sessions consisted of three trials per week following HT treatment, and the mean latency of three daily trials was reported. A trial ended when the rat fell or the maximum time elapsed [15].

Beam walking test: The setup featured a horizontal wooden beam, 100 cm long and 3 cm wide, elevated 30 cm above the floor, with safety platforms measuring 40 cm^2 at both ends**.** Rats (n = 12 per group) were trained to traverse the beam successfully before RES administration. Successful learning was defined as three consecutive traversals without falling. After HT treatment, the distance traveled and time to cross the beam were recorded in three consecutive trials. Falls were measured as the distance from the starting point to the point of fall [15].

Cognitive Tests

Working memory (Y-Maze Test): The Y-maze test involved 12 rats per group to evaluate behavior. The maze consisted of three identical arms measuring 40 × 30 × 15 cm, all radiating from a central point. Each rat was placed in one arm and allowed to explore for 5 minutes. An entry was recorded when a rat entered all four paws and an arm. Spontaneous alternation was defined as successive entries into all three arms in overlapping triplets. The alternation percentage was calculated as: Alternation (%) = [Number of successful alternations / (Total arm entries − 2)] × 100. Successful alternations occurred when the rat entered three different arms sequentially without repeats [16].

Passive Avoidance Memory: Passive avoidance memory was assessed using a shuttle-box apparatus (Borj Sanat, Iran) with two equal compartments (light and dark) separated by a guillotine door. The floor consisted of steel rods connected to an electrical circuit that delivered controlled shocks. The test involved three phases: 1. Habituation: Rats were placed in the light compartment for 1 minute. After 30 seconds, the door was opened, allowing the rat to enter the dark compartment freely. 2. Acquisition: Conducted 24 hours later. Rats were placed in the light compartment for 30 seconds, then allowed to enter the dark compartment. Once fully inside, the door was closed, and a foot shock (2 mA, 2 seconds, 50 Hz) was administered. Rats were removed 20 seconds later and returned to their home cages. 3. Retention: Conducted 24 and 48 hours after acquisition. Rats were placed in the light compartment for 30 seconds, then the door was opened without delivering a shock. Latency to enter the dark compartment (LDR) and total time spent in the dark (TDR) were recorded, with a maximum cutoff of 300 seconds. Longer LDR values indicated better retention of passive avoidance memory [17].

Measurement of Oxidative Stress Biomarkers and Pro-/Anti-inflammatory Cytokines

At the end of the treatment period, six animals from each group were deeply anesthetized with an intraperitoneal injection of 0.1 ml of 3% sodium pentobarbital, then decapitated with a rodent guillotine. Their brains were quickly removed and kept on ice. The right hippocampus (~30 mg) was carefully dissected under a stereomicroscope (Olympus, Japan). The tissue samples were rinsed with normal NS containing Tris buffer (Sigma, Germany) and homogenized for 5 minutes at 5000 rpm using a homogenizer (IKA, Germany). The homogenates were centrifuged in a refrigerated centrifuge (Hermle, Germany), and protease activity was halted by adding 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Germany) [18]. The supernatant was then collected for biochemical analysis.

Protein concentration was measured with a BCA assay kit (Thermo Fisher Scientific, A101251). Oxidative stress markers included: 8-hydroxy-2′-deoxyguanosine (8-OHdG), a DNA oxidative damage indicator, quantified by ELISA (Stat Fax, USA) using a commercial kit (Padgin Teb, Iran, ZB-11436C-H9648); carbonyl protein (CP), reflecting protein oxidation, measured with a kit (Kiazist, Iran, KCAR96); and 4-Hydroxynonenal (4-HNE), a lipid peroxidation product, assessed via a kit (Finetest, China, ER1587). Levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and the anti-inflammatory cytokine IL-10 were determined using ELISA kits (Karmania Pars Gene, Iran) as per the manufacturer's instructions. Optical density readings were taken at 450 nm [18].

Measurement of Cholinergic Activity (Acetylcholinesterase – AChE)

AChE activity in hippocampal tissue was assessed using Ellman's method, which measures the hydrolysis rate of acetylcholine. In brief, 0.4 ml of hippocampal homogenate supernatant was combined with 2.6 ml of phosphate buffer

(0.1 M, pH 8). Then, 0.1 ml of DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) was added, followed by 0.1 ml of acetylthiocholine iodide as the substrate. Absorbance was measured at 412 nm using a spectrophotometer, with readings taken over a 2-minute period. The enzyme activity was expressed as µmol of acetylcholine hydrolyzed per minute per mg of protein (µmol/min/mg protein) [19].

RT-qPCR Analysis of JNK/c-Jun/AP-1 Signaling Pathway

Total RNA was isolated from the left hippocampus (n = 6, approximately 30 mg) using a column-based RNA extraction kit (FavorPrep™ Tissue Total RNA Mini Kit). RNA quality was assessed by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Lite, Thermo Scientific, USA). The extracted RNA was stored at −80 °C until use. cDNA synthesis was carried out using RevertAid™ M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, K1621). For cDNA preparation, 1000 ng of RNA was combined with 0.5 µl of random hexamer primers and 0.5 µl of oligodT primers in a final volume of 12 µl of DEPC-treated water. Gene-specific primers for JNK, c-Jun, AP-1, and the endogenous control TATA-binding protein (TBP) (SinaPure™ ONE, Iran) were designed (Table 1). Real-time PCR was performed using the Applied Biosystems® StepOne™ system. All reactions were conducted in triplicate. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen), with control samples serving as the reference [20].

Histopathological Evaluation (Dark Neuron Density)

Following behavioral assessments, six animals per group were randomly chosen for histopathological analysis of dark neurons in the hippocampal CA1 and CA3 regions. Animals were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg). Transcardial perfusion was performed with cold NS followed by 4% paraformaldehyde. Brains were carefully removed and processed using an automated tissue processor (Autotechnicon). Paraffin blocks were prepared, and coronal sections (15 µm) of the hippocampus were obtained according to the stereotaxic atlas of Paxinos and Watson. Sections were stained with 1% toluidine blue. Dark neuron density was quantified using systematic random sampling and the disector technique. Numerical density (NA) was calculated using the stereological formula.

$$NA = \Sigma Q^{-} / (\Sigma P \times a \times h)$$

Where: NA = numerical density of neurons per unit volume, ΣQ^{-} = total number of dark neurons counted in dissectors, ΣP = total number of disector frames analyzed, a = area of the reference frame, h = height of the disector (distance between consecutive sections or effective section thickness).

At least 10 sections per animal (50 sections per group) were randomly selected for counting [19].

Statistical Analysis

Data analysis was conducted with SPSS version 26. Normal distribution was checked using the Kolmogorov-Smirnov test, while Bartlett's test was used to assess homogeneity of variances. Differences among groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. Results were considered statistically significant at $p < 0.05$.

Table 1. Primer Sequences

Genes	Primer Sequences	Sizes (bp)
TBP	*Forward: 5'- GCGGGGTCATGAAATCCAGT-3'*	*147*
	Reverse: 5'- AGTGATGTGGGGACAAAACGA -3'	
JNK	*Forward: 5'- CCAACCTCTCTGCTGCATTAT-3'*	*117*
	Reverse: 5'- GTGTCTATTCCTACAACCAGACG -3'	
C-JUN	*Forward: 5'- CGGAGATTCTACATTCACAGTCC-3'*	*92*

	Reverse: 5'- CATCATAAGCTGCACACACTATTC-3'	
AP-1	*Forward: 5'- AGATACGCTCCAAGCGGAGA -3'*	*180*
	Reverse: 5'- TCCAGGGAGGTCACAGACAT -3'	

Results

Motor Tests: Catalepsy, Rotarod, and Beam Walking

Statistical analysis, using one-way ANOVA followed by Tukey's post hoc test, revealed significant differences among the experimental groups in all three motor tests ($p < 0.001$). Specifically, the duration of cataleptic behavior (A) and the time spent on the wooden beam in the Beam Walking test (C) were significantly longer in the RES + NS and RES + HT groups compared to the control group ($p < 0.001$). Conversely, latency to fall in the Rotarod test (B) was significantly shorter in the RES + NS and RES + HT groups compared to the RES + NS group ($p < 0.001$).

Furthermore, the HT + VR and RES + HT groups showed significant reductions in cataleptic duration ($p < 0.001$) and Beam Walking performance ($p < 0.05$) compared to the RES + NS group, while experiencing notable improvements in Rotarod performance ($p < 0.001$). Additionally, significant differences were found between the RES + HT and HT + VR groups across all three motor tests ($p < 0.001$, Fig. 1).

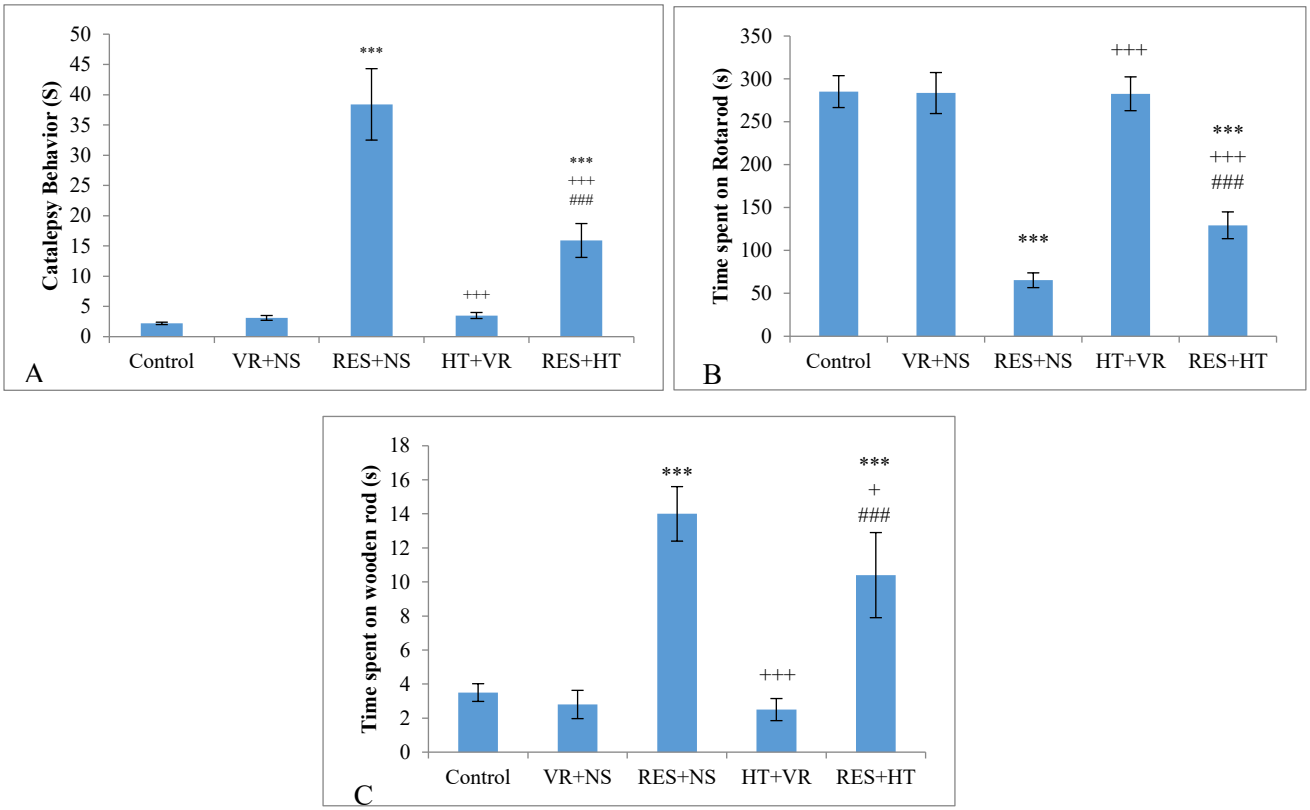


Figure 1 Comparison of mean ± SD values for catalepsy duration (A), rotarod performance (B), and beam walking latency (C) across experimental groups. Significant differences were observed between the control group and both RES + NS and RES + HT groups in all three motor tests (***p < 0.001). Furthermore, the HT + VR and RES + HT groups showed significant differences compared to the RES + NS group (+++p < 0.001). The RES + HT group also exhibited significantly higher values than HT + VR (###p < 0.001).

Cognitive Tests: Working Memory and Passive Avoidance

Statistical analysis of spontaneous alternation percentage (Fig. 2) using one-way ANOVA followed by Tukey's post hoc test showed significant differences among groups ($p < 0.001$). Specifically, spontaneous alternation in the RES + NS group was significantly lower compared to the control group ($p < 0.001$). In contrast, both the HT + VR and RES + HT groups showed highly significant increases compared to the RES + NS group ($p < 0.001$, Fig. 2A).

During the acquisition phase, there were no differences among groups in latency to dark room (LDR) or time spent in dark room (TDR) (Fig. 2B, 2C). However, at 24 and 48 hours after the shock, both measures showed significant changes ($p < 0.001$). Tukey's post hoc test revealed that the RES + NS group had significantly shorter LDR ($p < 0.001$) and longer TDR ($p < 0.001$) compared to controls. Similarly, the RES + HT group demonstrated reduced LDR at 24 hours ($p < 0.01$) and 48 hours ($p < 0.05$) after the shock, along with increased TDR at 24 hours ($p < 0.01$).

Conversely, the HT + VR and RES + HT groups showed significant increases in LDR ($p < 0.001$) and decreases in TDR ($p < 0.001$) compared to the RES + NS group at both time points. Notably, significant differences were also found between the HT + VR and RES + HT groups in LDR at both 24 h and 48 h ($p < 0.05$), as well as in TDR at 24 h post-shock ($p < 0.01$, Fig. 2B, 2C).

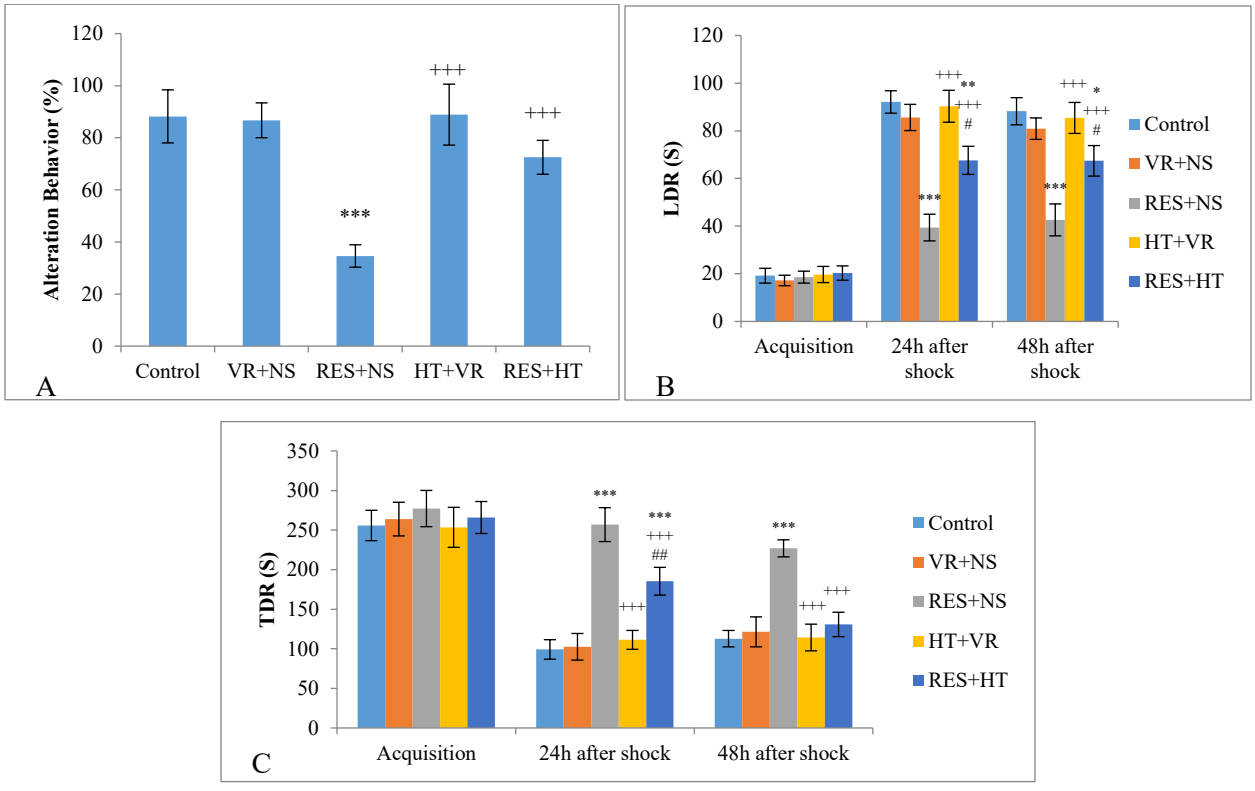


Figure 2 Comparison of mean ± SD values for spontaneous alternation percentage (A), latency to dark room (LDR, B), and time spent in dark room (TDR, C) across experimental groups. Significant differences were observed between RES + NS and RES + HT groups compared to the control group (***p < 0.001, **p < 0.01, *p < 0.05). Additionally, HT + VR and RES + HT groups showed significant increases in spontaneous alternation and LDR, along with significant decreases in TDR compared to RES + NS (+++p < 0.001).

Oxidative Stress Biomarkers

Hippocampal levels of 8-OHdG (Fig. 3A), CP (Fig. 3B), and 4-HNE (Fig. 3C) were significantly higher in the RES + NS group compared with controls (p < 0.001). Similarly, the RES + HT group showed significant increases in 8-OHdG (p < 0.05), CP (p < 0.001), and 4-HNE (p < 0.001) compared to the control group.

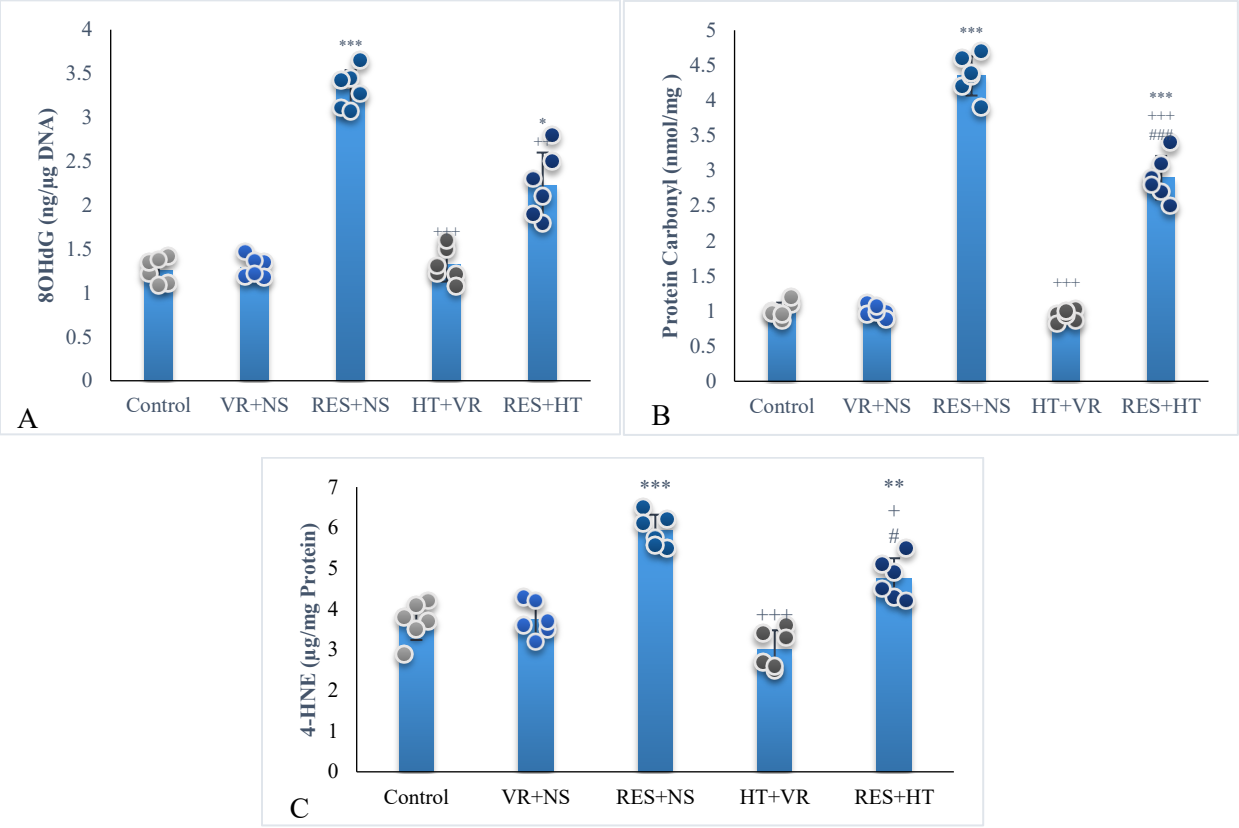


Figure 3 Comparison of mean ± SD values of hippocampal 8-OHdG (A), CP (B), and 4-HNE (C) across study groups. Both RES + NS and RES + HT groups showed significant increases in all three biomarkers compared to the control group (***p < 0.001, **p < 0.01, *p < 0.05). In contrast, HT + VR and RES + HT groups exhibited significant reductions in these parameters relative to RES + NS (+++p < 0.001, ++p < 0.01, +p < 0.05). Notably, CP and 4-HNE levels were significantly higher in RES + HT compared with HT + VR (### p < 0.001, # p < 0.05).

Pro- and Anti-inflammatory Cytokines

Hippocampal cytokine levels showed significant differences among groups ($p < 0.001$, Fig. 4). TNF-α (Fig. 4A), IL-1β (Fig. 4B), and IL-6 (Fig. 4C) were significantly elevated in the RES + NS group compared to controls ($p < 0.001$), while IL-10 (Fig. 4D) was significantly decreased ($p < 0.001$).

Both HT + VR and RES + HT groups showed significant decreases in TNF-α, IL-1β, and IL-6 compared to RES + NS ($p < 0.001$). Meanwhile, IL-10 levels were significantly higher in the HT + VR and RES + HT groups compared to the RES + NS group ($p < 0.001$). Notably, IL-6 and IL-10 levels in RES + HT differed significantly from those in HT + VR ($p < 0.01$ and $p < 0.05$, respectively).

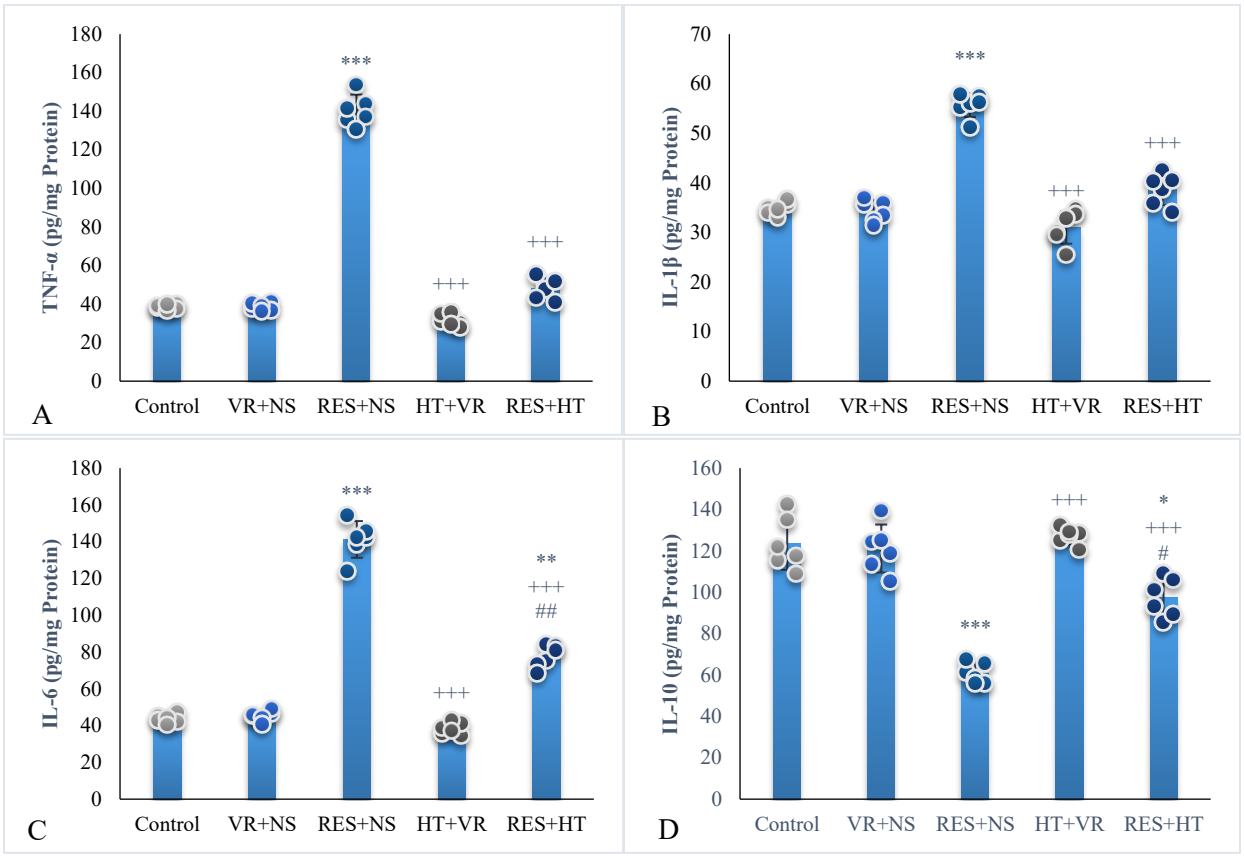


Figure 4 Comparison of mean ± SD values of hippocampal TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) across study groups. Significant differences were observed between the RES + NS and RES + HT groups compared with controls (***p < 0.001, **p < 0.01, *p < 0.05). Additionally, the HT + VR and RES + HT groups showed significant differences compared with the RES + NS group (+++p < 0.001). Notably, IL-6 and IL-10 levels differed significantly between the HT + VR and RES + HT groups (##p < 0.01, #p < 0.05).

Acetylcholinesterase (AChE) Activity

One-way ANOVA followed by Tukey's post hoc test revealed significant differences in hippocampal AChE activity among groups ($p < 0.001$). AChE activity was significantly higher in RES + NS and RES + HT groups compared with controls ($p < 0.001$ and $p < 0.01$, respectively). In contrast, the HT + VR and RES + HT groups showed significant reductions in hippocampal AChE activity compared with RES + NS ($p < 0.001$, Fig. 5).

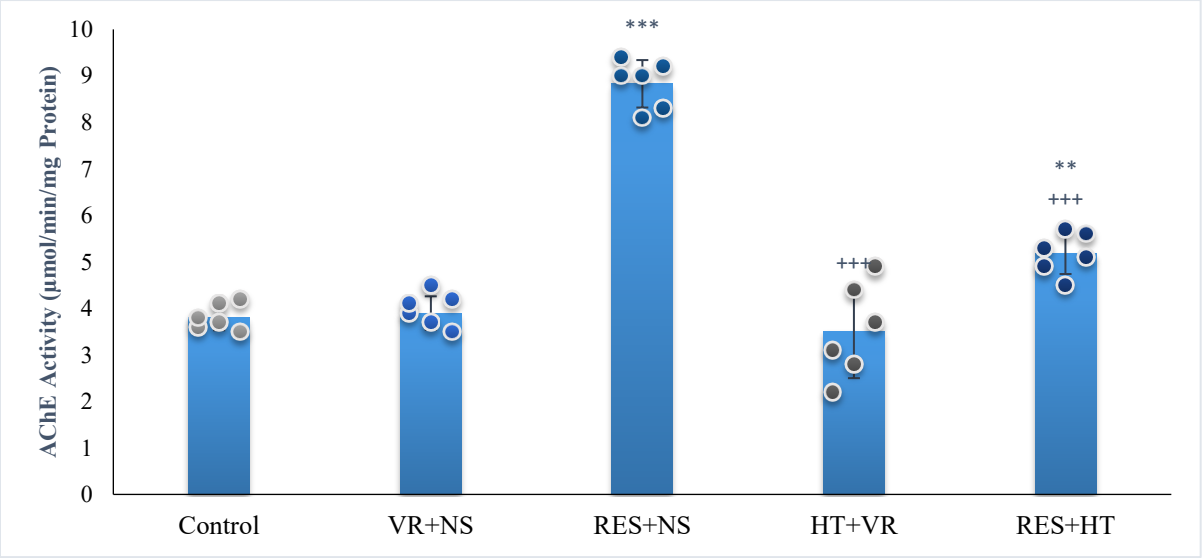


Figure 5 Mean ± SD values of hippocampal AChE activity across study groups. AChE activity was significantly higher in RES + NS and RES + HT groups compared to controls (**p < 0.01, ***p < 0.001). In contrast, HT + VR and RES + HT groups showed significant reductions in AChE activity compared to RES + NS (+++p < 0.001).

Gene Expression of JNK, c-Jun, and AP-1

One-way ANOVA followed by Tukey's post hoc test showed significant differences in mRNA levels of JNK (Fig. 6A), c-Jun (Fig. 6B), and AP-1 (Fig. 6C). JNK levels were significantly higher in RES + NS ($p < 0.001$) and RES + HT ($p < 0.05$) groups compared to controls. However, relative to RES + NS, JNK expression was significantly lower in the HT + VR ($p < 0.001$) and RES + HT ($p < 0.05$) groups, with notable differences also seen between the HT + VR and RES + HT groups ($p < 0.05$).

Similarly, c-Jun expression was significantly higher in RES + NS compared to controls ($p < 0.001$), but significantly lower in HT + VR ($p < 0.001$) and RES + HT ($p < 0.05$) groups relative to RES + NS. AP-1 expression was significantly increased in RES + NS ($p < 0.001$) and RES + HT ($p < 0.01$) groups compared to controls. Conversely, both the HT + VR ($p < 0.001$) and RES + HT ($p < 0.05$) groups showed significant decreases in AP-1 expression compared to the RES + NS group, with further differences observed between the HT + VR and RES + HT groups ($p < 0.05$).

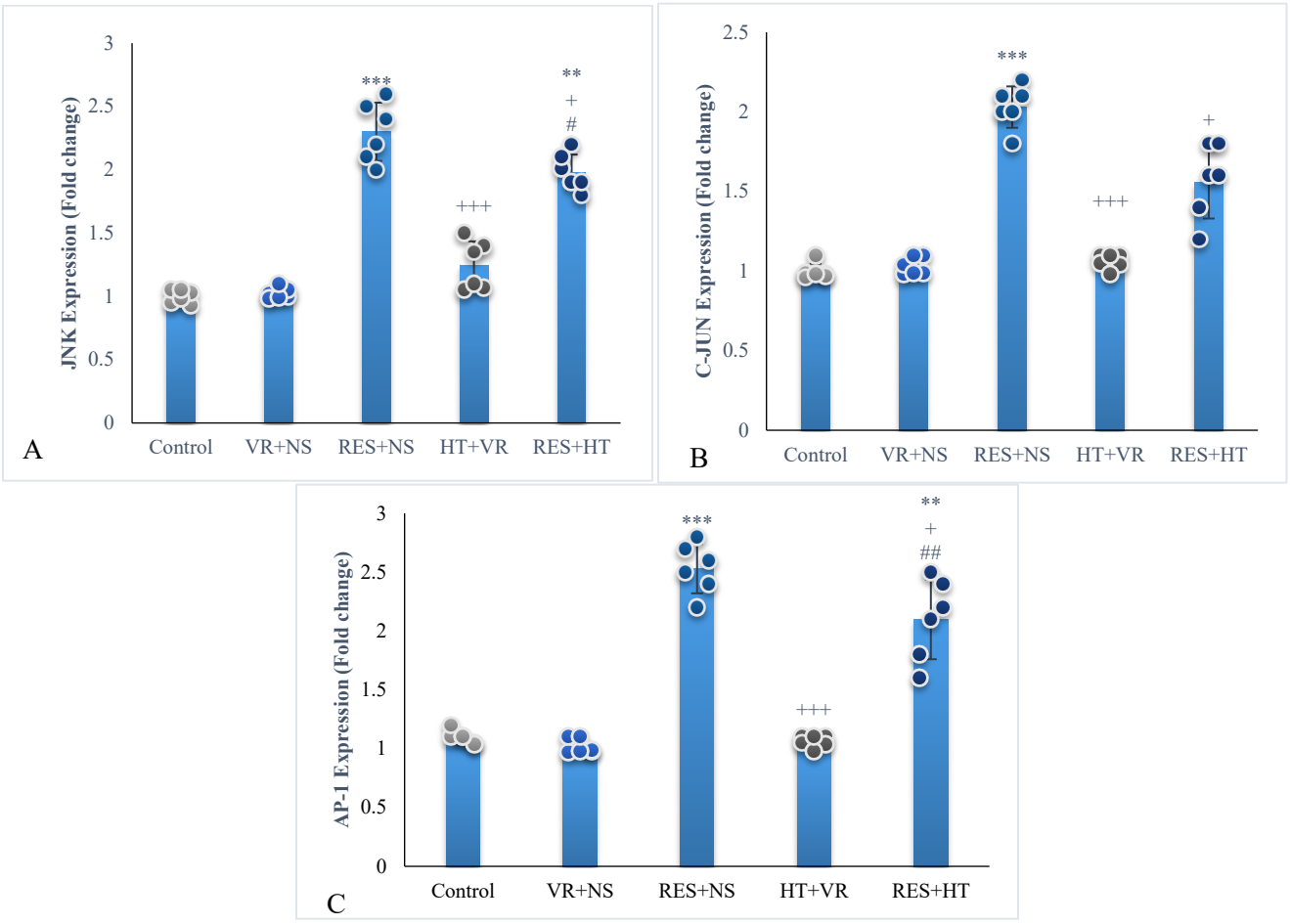


Figure 6 Mean ± SD values of hippocampal gene expression for JNK (A), c-Jun (B), and AP-1 (C) across study groups. Compared with controls, RES + NS and RES + HT groups showed significant increases in JNK and AP-1 expression, while RES + NS exhibited elevated c-Jun expression (***$p < 0.001$, **$p < 0.01$). In contrast, both HT + VR and RES + HT groups demonstrated significant reductions in all three genes relative to RES + NS (+++$p < 0.001$, +$p < 0.05$).

Hippocampal Dark Neuron Density

Toluidine blue staining showed increased dark neuron density in the RES + NS group compared to controls across hippocampal regions (Fig. 7A). One-way ANOVA revealed significant differences among groups in CA1 and CA3 regions ($p < 0.001$). Tukey's post hoc test confirmed that dark neuron density in CA1 (Fig. 7B) and CA3 (Fig. 7C) was significantly higher in RES + NS than in controls ($p < 0.001$). Additionally, the RES + HT group showed a significant increase in CA1 dark neuron density compared to controls ($p < 0.001$). However, both the HT + VR and RES + HT groups exhibited significant reductions in dark neuron density in CA1 and CA3 compared with RES + NS ($p < 0.001$). Notably, dark neuron density in CA1 differed significantly between the HT + VR and RES + HT groups ($p < 0.01$).

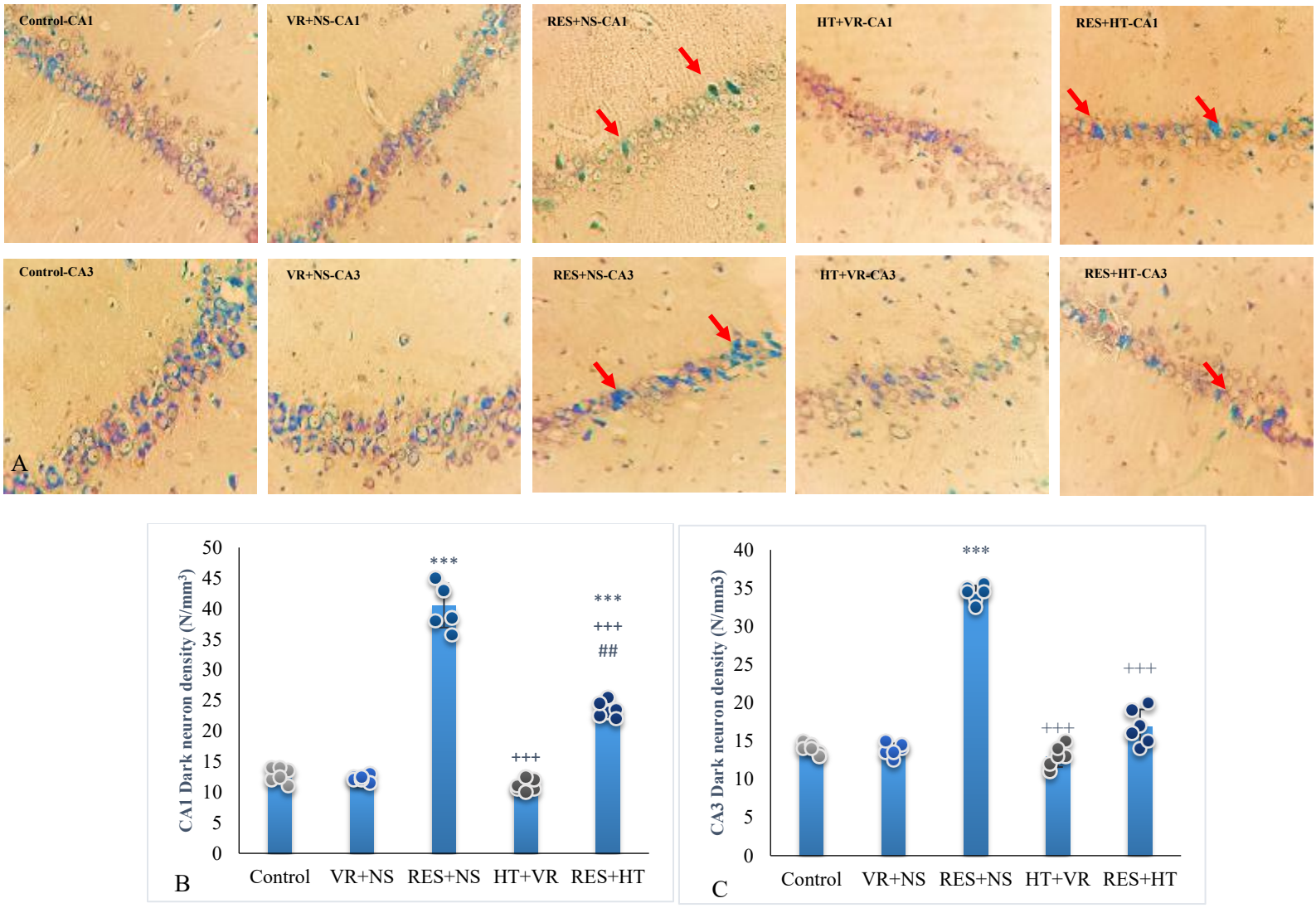


Figure 7 (A) Photomicrographs of hippocampal CA1 and CA3 regions across study groups, stained with toluidine blue (magnification ×40). An increase in dark neuron density was evident in the RES + NS group compared with controls, while a marked reduction was observed in the RES + HT group compared to RES + NS. (B, C) Comparison of mean ± SD values of dark neuron density in CA1 and CA3 regions. Both RES + NS and RES + HT groups showed significantly higher dark neuron density compared with controls (***$p < 0.001$). Conversely, HT + VR and RES + HT groups exhibited significant reductions in dark neuron density relative to RES + NS (+++$p < 0.001$). Notably, dark neuron density in CA1 differed significantly between HT + VR and RES + HT groups (##$p < 0.01$).

Discussion

The findings of this study showed that HT, the main polyphenol in extra-virgin olive oil, has significant neuroprotective effects in the RES-induced model of PD [21]. HT enhanced hippocampal neuronal function by decreasing oxidative stress, modulating inflammatory responses, and inhibiting the JNK–c-Jun–AP-1 signaling pathway. The improvements in motor and cognitive performance observed after HT treatment align with previous reports on the antioxidant and anti-inflammatory properties of olive oil polyphenols, confirming the key role of HT in reducing oxidative stress and neuroinflammation [22].

The reduction in oxidative damage markers of DNA, protein, and lipids in HT-treated groups suggests its ability to inhibit the generation of reactive oxygen species (ROS) and preserve cellular integrity. Behavioral improvements in

rotarod, Beam Walking, and Y-maze tests further indicate that HT not only alleviates motor symptoms but also ameliorates cognitive impairments induced by RES. This is particularly important since conventional PD therapies primarily target motor symptoms and often fail to address non-motor complications [23].

Mechanistically, suppression of the JNK–c-Jun–AP-1 pathway by HT was a key finding. This pathway regulates the expression of pro-inflammatory and pro-apoptotic genes, and its hyperactivation in PD contributes to neuronal death and cognitive decline [24]. RT-qPCR results confirmed that HT downregulated genes associated with this pathway, shifting cellular signaling toward survival and repair. This mechanistic insight explains the observed decrease in dark neuron density in hippocampal CA1 and CA3 regions. Together, these findings highlight the crucial role of HT in regulating inflammation and promoting neuroprotection, in line with previous research on olive oil polyphenols [25].

RES administration significantly increased hippocampal pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) while reducing the anti-inflammatory cytokine IL-10, underscoring the hippocampus as a key site of neuroinflammatory responses in PD pathogenesis [26]. These results align with earlier studies that reported central inflammation and cytokine dysregulation in PD models [27]. Importantly, HT, with its ability to cross the blood–brain barrier, has been shown to suppress hippocampal inflammation in PD models [28]. By limiting pro-inflammatory and pro-apoptotic gene expression while enhancing anti-inflammatory cytokines such as IL-10 [29], HT reduces microglial overactivation, prevents synaptic damage, and improves cognitive performance. Its effects extend to mitochondrial preservation and neurotrophic signaling (BDNF/TrkB/CREB), further supporting its role in neuroprotection [30]. Additionally, HT prevents toxic protein aggregation (e.g., α-synuclein), reduces ROS, inhibits NF-κB and JNK phosphorylation, and suppresses NLRP3 inflammasome activation, thereby attenuating neuroinflammation [31].

The modulation of pro- and anti-inflammatory cytokine levels highlights HT's capacity to restore neuroimmune balance. It can be hypothesized that HT, by creating an anti-inflammatory environment in the hippocampus, not only protects dopaminergic neurons responsible for motor function but also prevents degeneration of neurons critical for memory and learning in PD patients. Nevertheless, variations in dosage, administration route, and experimental models complicate direct comparisons across studies. Furthermore, it remains unclear whether the observed hippocampal effects are due to local actions of HT or systemic modulation of inflammatory responses.

The JNK–c-Jun–AP-1 pathway, as part of the MAPK signaling network, plays a central role in neuronal and glial responses to oxidative stress and inflammation. Its hyperactivation in neurodegenerative diseases, such as PD, leads to increased transcription of pro-inflammatory and apoptotic genes [32]. Under pathological conditions, oxidative stress from α-synuclein aggregation and ROS production phosphorylates JNK, which activates c-Jun and forms the AP-1 complex, driving transcription of TNF-α, IL-1β, IL-6, and apoptotic mediators such as Bax. These events collectively exacerbate neuroinflammation and neuronal death in hippocampal regions.

HT counteracts this by downregulating ASK1 (an upstream activator of JNK), activating the Nrf2/ARE pathway, enhancing antioxidant enzymes (HO-1, NQO1, GST), directly inhibiting MAPKs (JNK and p38), activating phosphatases (DUSP1, DUSP10), and promoting rapid dephosphorylation of JNK and c-Jun. It also reduces cytokines such as TNF-α and IL-1β that activate JNK, thereby modulating this pathway [33]. Given the hippocampus's central role in neurochemical and cognitive regulation, modulation of JNK–c-Jun–AP-1 by HT may have profound effects on preserving neuronal integrity and non-motor functions in PD models.

Despite these promising results, several limitations must be acknowledged. The RES model reflects only part of PD's complex pathophysiology and cannot fully replicate human conditions. Molecular analyses were restricted to the JNK–c-Jun–AP-1 pathway, while other pathways related to inflammation, apoptosis, and autophagy warrant further investigation. Moreover, clinical studies are needed to determine the optimal dosage, duration, and safety of HT in humans.

Conclusion

In summary, this study demonstrated that HT, through its antioxidant and anti-inflammatory properties, significantly improved motor and cognitive functions in a RES-induced PD model. Suppression of the JNK–c-Jun–AP-1 pathway and reduction of oxidative stress markers were key mechanisms underlying these effects. HT thus emerges as a

promising adjunct therapeutic strategy for mitigating neuroinflammation and preserving cognitive function in PD. Future clinical trials are essential to confirm its safety and effectiveness in humans, which could offer a new comprehensive approach for PD treatment.

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Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. All procedures adhered to international ethical guidelines and were approved by the Ethical Committee for Laboratory Animals at Islamic Azad University, Shiraz Branch (Ethics Code: IR.IAU.SHIRAZ.REC.1404.266).

Research Highlights

- HT significantly improved motor and cognitive performance in a RES-induced Parkinson’s model.
- HT reduced oxidative stress biomarkers (8-OHdG, CP, 4-HNE) and restored hippocampal cytokine balance.
- Suppression of the JNK–c-Jun–AP-1 pathway by HT promoted neuronal survival and reduced dark neuron density.
- Findings highlight HT as a natural adjunct therapy with dual antioxidant and anti-inflammatory actions in PD.