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Targeting AT_1 Receptors with Telmisartan Protects SH-SY5Y Cells from 6-OHDA Induced Parkinsonian Neurotoxicity

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by oxidative stress, neuroinflammation, and α-synuclein accumulation. Activation of the angiotensin II type 1 (AT_1) receptor contributes to these pathogenic mechanisms. This study aimed to evaluate the neuroprotective effects of telmisartan, an AT_1 receptor antagonist, in a cellular model of PD.

An in vitro PD model was established by exposing SH-SY5Y neuroblastoma cells to 200 µM 6-hydroxydopamine (6-OHDA). Cells were pre-treated with varying concentrations of telmisartan prior to toxin exposure. Cell viability was assessed using MTT and LDH assays. Oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), were measured. Inflammatory mediators and apoptotic markers were analyzed, and α-synuclein and IL-1β expression were evaluated by immunofluorescence.

Telmisartan pre-treatment significantly improved cell viability. While 6-OHDA increased MDA levels and reduced antioxidant enzyme activities, telmisartan reversed these effects by attenuating oxidative stress. In addition, telmisartan downregulated pro-inflammatory mediators (TNF-α, IL-6, NF-κB, and caspase-3) and increased IL-10 expression. Immunofluorescence analysis showed that telmisartan suppressed 6-OHDA-induced α-synuclein and IL-1β overexpression.

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These findings indicate that telmisartan protects SH-SY5Y cells against 6-OHDA-induced neurotoxicity and supports AT_1 receptor blockade as a promising neuroprotective strategy for Parkinson's disease.

Keywords: 6-OHDA; AT1 receptor; Parkinson's disease; SH-SY5Y cells; telmisartan

1. Introduction

Parkinson's disease (PD) stands as one of the prevalent age-related neurodegenerative disorders, affecting over 6 million individuals worldwide, comprising 2-3% of the elderly population. PD is pathologically characterized by damaged dopaminergic neurons with existence of Lewy bodies and aggregation of α-synuclein in the substantia nigra [1]. Clinical hallmarks of the PD include motor disabilities with resting tremor, rigidity, akinesia, bradykinesia and postural abnormalities [2].

The most important treatment option for initial phase PD consists of various medications, such as L-DOPA While this treatment approach does not halt disease progression, long-term L-DOPA therapy is associated with the development of involuntary motor complications, including dyskinesia[3].

Specific mechanisms causing irreversible neuronal impairment in PD have not been fully identified, but there are many suspected mechanisms including neuroinflammation [4], mitochondrial dysfunction [5], neuronal death [6], disturbed redox status [7] and protein misfolding [8]. Identifying a

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suitable agent capable of modulating this complex network of mechanisms makes it crucial to establish a therapeutic modality.

Angiotensin-(1–7) has a notable role in the pathophysiology of various neurodegenerative disorders, including PD. Renin-angiotensin system (RAS) has been considered amongst the most crucial key neuroendocrine components implicated in blood pressure management [9]. However, recent evidence suggests that the presence of a local RAS in multiple organs is associated with diverse physiological and pathophysiological roles. Angiotensin II and the angiotensin II type 1 receptor are key activators of the local nigrostriatal renin–angiotensin system in the brain. Activation of the AT_1 receptor stimulates the microglial NADPH oxidase complex, leading to increased neuroinflammatory responses and contributing to the pathogenesis of PD and other neurodegenerative disorders [10-12].

Nigrostriatal local RAS activation implicated in the modulation of dopaminergic neurotransmission, blood flow, and proinflammatory pathway which generates ROS leading to dopaminergic cell death [13, 14]. AT1-activation aggravates the microglial inflammation, oxidative stress, and neuronal dysfunction in the 6-OHDA-induced PD models [15-17]. One of the most effective AT1 blockers, telmisartan (TLM) is an antihypertensive agent which was shown to have neuroprotective effects in PD in mouse models [18, 19].

Therefore, this study was designed to investigate the effects of TLM in a 6-OHDA-induced PD model in SH-SY5Y cells, with a particular focus on its potential to inhibit α-synuclein accumulation by alleviating autophagic dysfunction, oxidative stress, and neuroinflammation.

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2. Materials and Methods

2.1. Cellular model

The undifferentiated human-derived SH-SY5Y neuroblastoma cell line (ATCC Cat. No. CRL-2266) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Experiments were performed using cells at passage 4. Cells were seeded at a density of 5×10^3 cells per well and pre-treated with TLM prior to 6-OHDA exposure. TLM was applied at four concentrations: TLM1 (0.05 µM), TLM2 (0.1 µM), TLM3 (0.5 µM), and TLM4 (1 µM).

SH-SY5Y cells are widely used as a cost-effective human neuroblastoma-derived in vitro model for PD research and can express human protein isoforms that may differ from those in rodent primary cultures [20, 21].

After 24 h of cell adhesion, SH-SY5Y cells were exposed to 200 µM 6-OHDA for 24 h at a density of 5×10^3 cells per well. The concentration of 200 µM 6-OHDA was selected based on previous studies demonstrating its ability to induce reproducible dopaminergic neurotoxicity in SH-SY5Y cells [22, 23]. Cells were pre-treated with TLM at 0.05 µM, 0.1 µM, 0.5 µM, and 1 µM for 30 min prior to 6-OHDA exposure. Cell viability was assessed after 24 h[22].

2.2. Cell viability assay

This assay serves the purpose of assessing cell viability. MTT solution (SigmaAldrich, Cas No: 298-93-1) was administered to each well following the kit's protocol. After incubation with MTT for 4 h, the formazan precipitate was solubilized in 150 µL of dimethyl sulfoxide (DMSO), and

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absorbance values were recorded at 490 nm using a microplate spectrophotometer (BioTek Instruments, USA) [22].

Cytotoxicity was evaluated by measuring lactate dehydrogenase (LDH) activity using a commercial LDH Activity Assay Kit (Elabscience, Cat. No. E-BC-K766-M), according to the manufacturer's instructions. Briefly, cell culture supernatants were collected after experimental treatments and centrifuged at 10,000 × g for 10 min at 4 °C to remove cellular debris. Fresh reaction working solution was prepared for each well by mixing substrate, chromogenic agent, and coenzyme working solution as recommended by the kit protocol. The reaction mixture was incubated under the conditions specified by the manufacturer and subsequently terminated using the provided stop solution. Absorbance was measured at 450 nm using a microplate reader.

2.3. Measurement of oxidative stress markers

The malondialdehyde (MDA) levels (Elabscience, USA, Cat. no. E-EL-0060), the activities of superoxide dismutase (SOD) (Elabscience, USA, Cat. no. E-BC-K019-M), catalase (CAT) (Elabscience, USA, Cat. no. E-BC-K031-M) and glutathione (GSH) (Elabscience, USA, Cat. no. E-BC-K030-S), were detected by ELISA kits in conformity with kit protocol [24]. Samples were prepared according to the manufacturers' instructions. Briefly, appropriate standards and samples were added to the ELISA plates and incubated at room temperature. After washing steps to remove unbound substances, enzyme-conjugated detection reagents were added, followed by substrate solution. The reaction was stopped, and absorbance was

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measured using a microplate reader at the wavelength specified by the manufacturer. The absorbance was read at 450 nm [25].

2.4. qRT-PCR analysis

mRNA extraction and cDNA synthesis procedures were conducted using the RNeasy easy kit (Qiagen, Hilden, Germany), as previously described [26]. The cDNA synthesis was accomplished using a High-Capacity cDNA Reverse Transcription Kit, following the kit protocol [27] . Quantitative real-time PCR (qRT-PCR) was performed on a Rotor-Gene Q system (QIAGEN) using TaqMan (Applied Biosystems, Thermo Fisher Scientific) chemistry as previously described [24]. The mRNA expression levels of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), nuclear factor- κB (NF-κB), IL-10, and caspase-3 were assessed using the Rotor-Gene Q system (QIAGEN) (Table 1). The relative expression levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method, normalized to the housekeeping gene β-actin [26, 28].

2.5. Immunofluorescence

Immunofluorescence was used to assess the aggregation of α-synuclein and IL-1β. After fixation, the cells were blocked in 5% BSA for 60 minutes after permeabilized with PBS containing 0.1% Triton-X-100 for 10 minutes. . Then, the samples were incubated with primary antibodies against α-synuclein (Santa Cruz, Cat. no. sc-69977) and IL-1β (Santa Cruz, Cat. no. sc-52012) (1:200) overnight at 4 °C. The cells were then washed three times with PBS and incubated at room temperature for 1 h with a goat anti-mouse FITC-conjugated secondary antibody (Jackson ImmunoResearch, Cat. No. 115–095–003) at dilution ratios of 1:100, 1:200,

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1:500, and 1:1000. The 1:100 dilution was selected for experiments, as it provided the highest immunopositive signal. Subsequently, the cells were washed three times with PBS and incubated with secondary antibody (1:100) for 1 hour at constant temperature. Finally, the cells were counterstained with 1 µg/ml of DAPI for 2 hours at 25°C and imaged with a fluorescence microscope (Zeiss). Fluorescent intensities were tested with the Fiji Image J Software, Version 2.9.0, URL: https://imagej.net/software/fiji/.

2.6. Statistical analyses

All analyses were performed by post hoc Tukey's test, following directional analysis of variance (one-way ANOVA) (IBM SPSS 22.0). ($p < 0.05$) [29]. Data were shown as mean ± SD.

3. Results

3.1. MTT and LDH assay

Figure 1 presents the results of MTT and LDH assays. In the MTT analysis, exposure to 6-OHDA led to a significant reduction in SH-SY5Y cell viability by 35.42%, while LDH release increased by 134.1%. Notably, pre-treatment with TLM in MTT analysis substantially enhanced cell viability, demonstrating an induction of cell proliferation by 32.62%, 56.61%, 47.9%, and 38.16% for TLM1, TLM2, TLM3, and TLM4, respectively, compared to the 6-OHDA group. Furthermore, in LDH analysis, TLM pre-treatment significantly attenuated LDH release by 19.16%, 43.70%, 40.61%, and 38.05% for TLM1, TLM2, TLM3, and TLM4, respectively, relative to the 6-OHDA group ($p<0.001$).

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3.2. Oxidative stress

Treatment with 200 μM 6-OHDA resulted in a 2.4-fold increase in MDA levels compared with the control group ($p < 0.001$) (Fig. 2). Moreover, 6-OHDA significantly suppressed SOD, CAT, and GSH activities in SH-SY5Y cells by 78.91%, 72.39%, and 73.08%, respectively.

TLM2 and TLM3 significantly attenuated 6-OHDA-induced MDA production by 53.39% and 41.17%, respectively ($p < 0.001$) (Fig. 2). Similarly, TLM2 significantly increased the activities of SOD, CAT, and GSH in 6-OHDA-exposed SH-SY5Y cells by 295.39%, 195.08%, and 178.43%, respectively, compared with the 6-OHDA group ($p < 0.001$).

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3.3. RT-PCR analysis

The release of cytokines was markedly upregulated compared with the control group, with the exception of IL-10 (Fig. 3). TLM2 significantly reduced the expression levels of TNF-α, IL-6, NF-κB, and caspase-3 by 53.04%, 53.38%, 39.56%, and 44.32%, respectively, compared with the 6-OHDA group, while significantly increasing IL-10 expression by 115.78%.

3.4. Immunofluorescence

Excessive accumulation of α-synuclein leads to neuronal toxicity and contributes to the progression of PD. As shown in Figures 4 and 5, α-synuclein and IL-1β were significantly overexpressed in 6-OHDA-exposed cells. Notably, TLM markedly attenuated the expression of both α-synuclein and IL-1β.

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4. Discussion

In the present study, we hypothesized that TLM exerts neuroprotective effects against 6-OHDA-induced neurotoxicity in SH-SY5Y cells by modulating oxidative stress, inflammatory responses, and apoptotic pathways. Our findings support this hypothesis by demonstrating that TLM markedly reduced oxidative burden, suppressed pro-inflammatory cytokine expression, inhibited apoptotic signaling, and decreased α-synuclein accumulation in a cellular model of PD. To our knowledge, this is the first study to demonstrate the protective role of TLM in an in vitro 6-OHDA-induced PD model, highlighting its potential as a multi-target therapeutic agent. A commonly used experimental PD model was formed with 6-OHDA in human neuroblastoma SH-SY5Y cells, and the protective role of TLM was investigated in this model for the first time.

6-OHDA is an analogue of dopamine that accumulates intracellularly in cells. This accumulation causes multiple forms of cellular damage, including oxidative stress, mitochondrial dysfunction, and apoptosis, ultimately leading to degeneration of catecholaminergic neurons, particularly dopaminergic neurons [30, 31]. It is often preferred to create a PD model due to its neurotoxic effects. Therefore, SH-SY5Y human neuroblastoma cells were exposed to 6-OHDA to induce neurotoxicity and to investigate the potential protective effects of TLM in an in vitro PD model.

Excessive angiotensin II activity is a crucial harmful factor which contributes to the initiation of neuroinflammation and exerts its effects primarily through AT1R and AT2R [32-34]. Activation of the AT1R has been

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linked to neurotoxicity and inflammatory responses in the brain [32-34]. AT1R overexpression has been reported to contribute to neurotoxicity by activating NADPH oxidase–derived ROS production and disrupting intracellular Ca^{2+} homeostasis. These changes potentiate oxidative stress and inflammatory signaling and thereby favor α-synuclein aggregation. Intricately, increased α-synuclein expression in PD has been reported to further enhance AT1R activity and elevate pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [35]. Blockade of the AT_1 receptor with antagonists such as TLM has been shown to reduce α-synuclein expression and accumulation in experimental models of PD, likely through the attenuation of NADPH oxidase–mediated oxidative stress and downstream inflammatory signaling pathways. Consistent with this mechanism, previous studies have demonstrated that AT_1 receptor antagonists reduce α-synuclein levels, suppress microglial activation, and provide neuroprotection in toxin-induced models of Parkinsonism[18, 35].

Using specific medications that block the angiotensin II AT1R helps reduce inflammation, cell damage, and cell death. This has been shown in various types of brain cells like neurons, blood vessel cells, microglia, and astrocytes. It's also evident in different animal models that simulate conditions such as inflammation, injuries, and brain disorders involving nerve cell degeneration. [33, 34, 36, 37]. We investigated the effects of TLM based on the hypothesis that TLM, an AT1 receptor antagonist, may exert protective effects in an in vitro PD model through its neuroprotective and anti-inflammatory properties.

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In this study, we first examined the antioxidant activity of TLM and subsequently demonstrated its ability to alleviate oxidative burden by reducing the elevated levels of ROS and MDA induced by 6-OHDA. As 6-OHDA-induced oxidative stress along with ROS and MDA elevation disrupts mitochondrial membrane integrity resulting in apoptotic cell death, inhibition of oxidative stress burden may portray a crucial contribution to defend neuronal integrity [38]. Neuronal cell death in neurodegenerative diseases, including PD, can occur in the pathological process of neuronal toxicity from various causes, as well as from over-supply of H2O2 related to oxidative stress. The antioxidant activity of TLM, characterized by increased SOD, CAT, and GSH levels and decreased ROS and MDA levels, may account for the attenuation of oxidative stress observed in 6-OHDA-exposed SH-SY5Y cells.

TLM protected SH-SY5Y cells against 6-OHDA-induced oxidative stress, which is associated with both apoptotic and necrotic cell death. It is evident that high concentrations of oxidative stress markers lead to apoptosis triggered by mitochondrial disruptions and production of apoptotic markers [39, 40]. Moreover, ROS intervene with intra-cellular signaling cascades and the release of diverse apoptotic factors, such as caspase-3 [41]. In this study, we demonstrated that TLM exhibits anti-apoptotic activity by lowering MTT and LDH levels and inhibiting caspase-3 activation induced by 6-OHDA.

In this context, TLM may attenuate 6-OHDA-induced neuronal cell death through the preservation of mitochondrial function. Among the factors that cause dopaminergic apoptosis in the neurons of PD patients

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are oxidative stress and mitochondrial dysfunction. Many studies have implied that oxidative stress induces inflammatory responses in microglia in neuro-inflammation in PD [42, 43]. Moreover, chronic activation of microglia intensifies the generation of oxidative stress. This process also promotes the release of multiple factors, such as proinflammatory cytokines, which ultimately trigger apoptotic pathways and inflammation in neurons [44]. Additionally, it has been proven in previous studies that apoptosis stimulates inflammatory responses during the development of PD [44].

Due to the counterbalance of pro and anti-inflammatory processes, inflammation also plays a biphasic role in PD disease. TLM alleviated 6-OHDA-induced inflammation by decreasing TNF-α and IL-6 levels while increasing IL-10 concentrations. In previous reports, it was demonstrated that, IL-6 concentrations of PD patients with higher motor disfunctions were increased [45]. This information seems to indicate that motor dysfunctions of PD patients may be related to increased IL-6 concentrations. In a previous model of PD in mice, increased IL-10 concentration was associated with decreased number of activated microglia and decreased TNF levels [46, 47]. Based on these findings, IL-10, in contrast to the pro-inflammatory cytokines TNF-α and IL-6, may act as an anti-inflammatory mediator that helps protect against PD progression and symptom severity.

According to our results, TLM suppressed the inflammatory process activated by 6-OHDA by decreasing TNF-□ and IL-6 levels and increasing IL-10 levels. Moreover, over-expression of IL-1β level was detected in our

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immunofluorescence experiments. Consistent with these findings, previous studies have demonstrated increased serum IL-1β levels in patients with PD [48].

It has also been claimed that elevated serum IL-1β predicts the onset of PD before the development of clinical symptoms [49]. α-synuclein is a presynaptic protein with unknown physiological function[50] and causes the formation of Lewy bodies, one of the important hallmarks of PD [51]. It is known that α-synuclein has a role in the aggravation of PD disease and can serve as a biomarker to detect PD [52]. Besides, data from immunofluorescence assays proved that the expression levels of α-synuclein were markedly up-regulated in the 6-OHDA-induced PD, while α-synuclein levels profoundly decreased with TLM treatment.

This study has some limitations inherent to the in vitro 6-OHDA-induced PD model. Although this system is useful for investigating PD-related cellular stress responses, it cannot fully recapitulate the complexity of PD in vivo, including systemic influences, and long-term progressive neurodegeneration. Furthermore, the use of a SH-SY5Y model without in vivo confirmation may limit the generalizability of these findings. Accordingly, future studies should validate the observed effects of TLM in appropriate in vivo PD models and expand outcomes to include nigrostriatal dopaminergic integrity and behavioral assessments.

5. Conclusion

In conclusion, TLM noticeably prevented 6-OHDA-induced neurodegeneration and apoptosis in SH-SY5Y-cells via alleviating oxidative burden, the inflammation, as well as decreasing the accumulation of □-

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synuclein. This study adds to the existing literature on the neuroprotective effects of blocking AT1 receptors and offers new insights into the therapeutic benefits of AT1 receptor blockade in PD.

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Figure Legends

Fig. 1. Effects of telmisartan on cell viability. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; #p < 0.05 and ##p < 0.001 vs. 6-OHDA group.

Fig. 2. Effects of telmisartan on oxidative stress parameters. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; ##p < 0.001 vs. 6-OHDA group.

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Fig. 3. qRT-PCR analysis results. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; #p < 0.05 and ##p < 0.001 vs. 6-OHDA group.

Fig. 4. Immunofluorescence staining of α-synuclein. Representative images from (A) control, (B) 6-OHDA, (C) TLM-1, (D) TLM-2, (E) TLM-3, and (F) TLM-4 groups (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; ##p < 0.001 vs. 6-OHDA group.

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Fig. 5. Immunofluorescence staining of IL-1β. Representative images from (A) control, (B) 6-OHDA, (C) TLM-1, (D) TLM-2, (E) TLM-3, and (F) TLM-4 groups (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; ##p < 0.001 vs. 6-OHDA group.

FIGURES

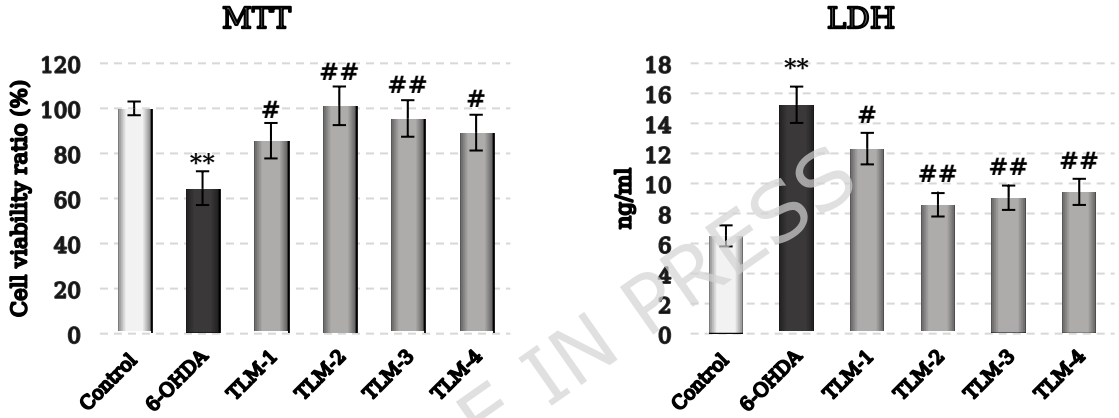


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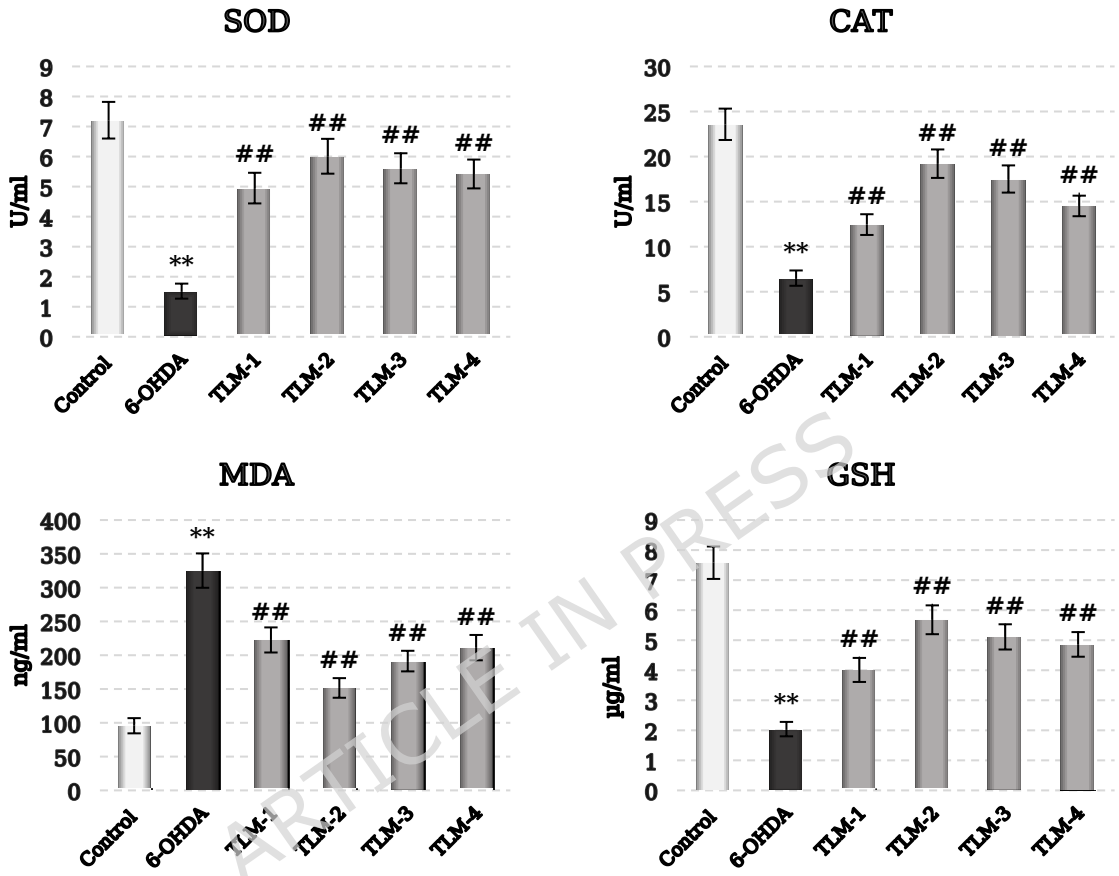
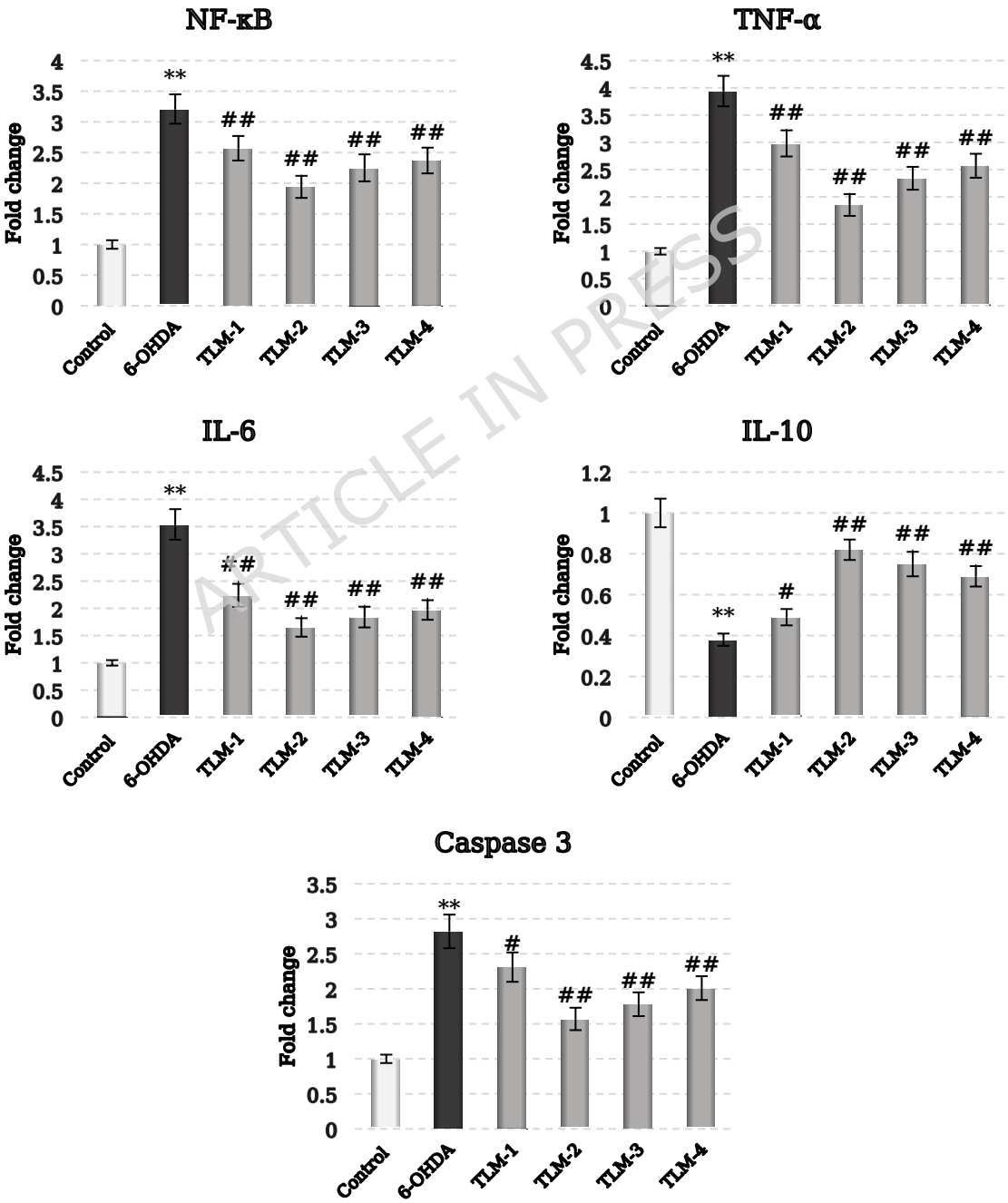


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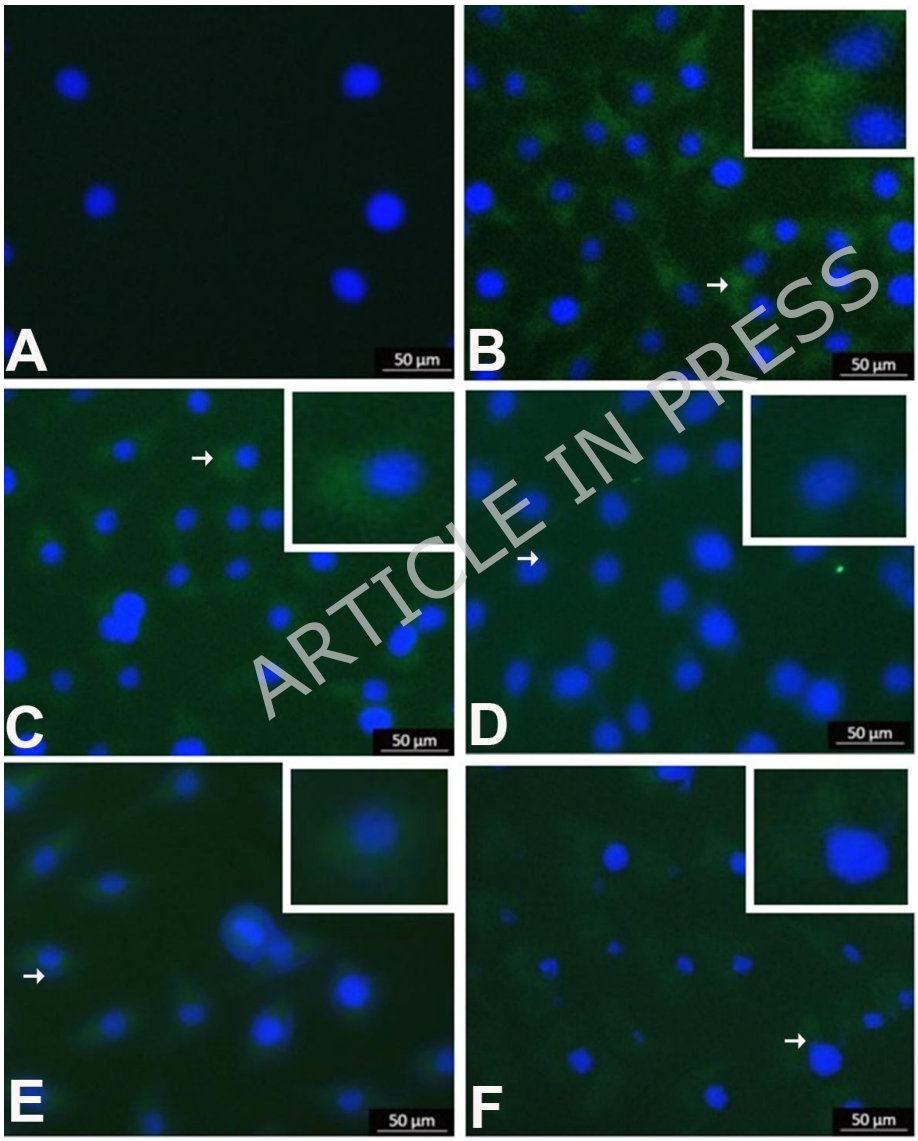
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Fig. 3. qRT-PCR analysis results. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **p < 0.001 vs. control group; #p < 0.05 and ##p < 0.001 vs. 6-OHDA group.



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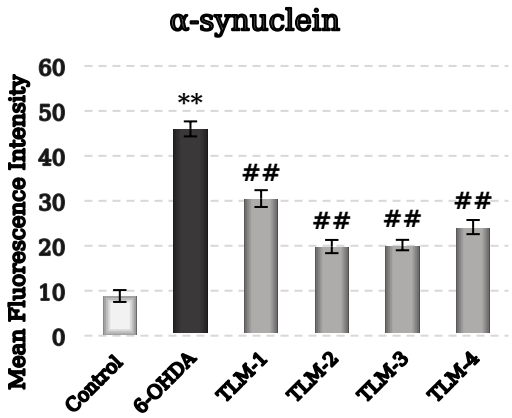
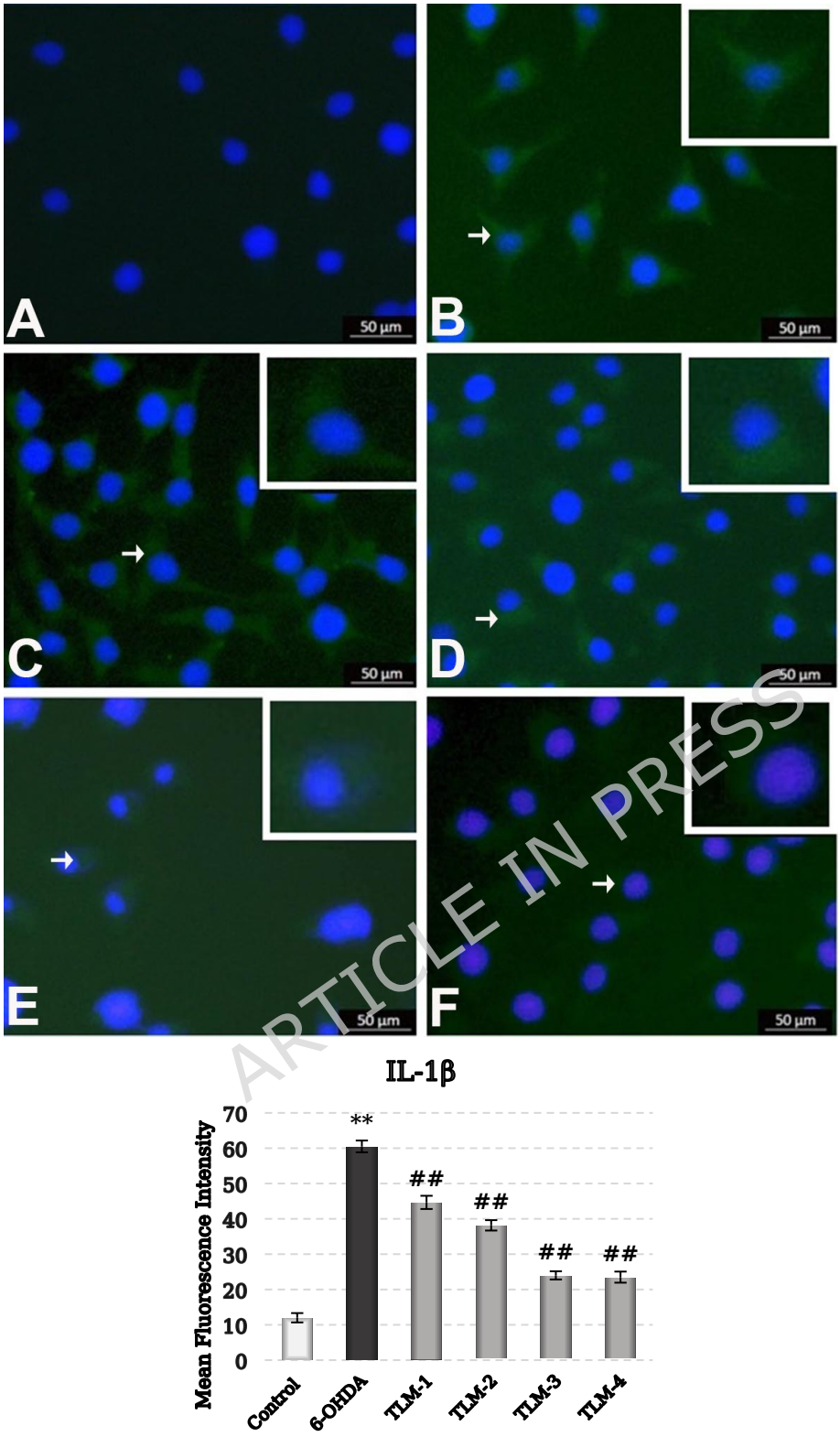


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TABLES

Table 1. List of the sequences of the primers used for reverse transcription real-time quantitative polymerase chain reaction (RT-PCR).

Genes	Forward Sequence (5’-3’)	Reverse Sequence (5’-3’)
IL-10	CCTCTGCTTAGAGCGTTTCC	TTTCTGCGGAGCTACATTCG
IL-6	TTCCTGCAGAAAAAGGTGGG	AGAGAGCCAACCAACCAAAC
NF-κB	CTCCAGGACACGGTGTTTTT	GATTTCCTCCGAAGCTGGAC
TNF-α	ATTCTGGGTTTGGGTTTGGG	CTACAACATGGGCTACAGGC
Caspase-3	TTTTCAGTCCGGGGACAAAC	GGGCAGCCGAGAATAACAAT

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