Research Article

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# Effects of The Antidepressant Venlafaxine on The Expression of Peroxiredoxin-3 and Peroxiredoxin-5 Against LPS-Induced Oxidative Stress in SH-SY5Y Neurons

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**ABSTRACT**

The main objective of this study was to examine the potential neuroprotective properties of venlafaxine, an antidepressant belonging to the serotonin-norepinephrine reuptake inhibitor class. Furthermore, it provides a novel insight into how venlafaxine might interact with peroxiredoxin-3 and -5 enzymes (PRDX-3 and PRDX-5), pivotal cellular antioxidant defence system components. For this purpose, human neuroblastoma cells (SH-SY5Y) were pretreated with venlafaxine (0-100 µM) for 12 h, followed by a 4 h LPS exposure (1 µg/mL) to induce oxidative stress. Cell viability was determined by MTT assay, and ROS generation was assessed by DCFH-DA assay. Protein and mRNA expression levels of PRDX-3 and PRDX-5 were determined by immunoblotting and qRT-PCR, respectively. Based on the results obtained, it was found that the venlafaxine pre-treatment led to a notable reduction in intracellular ROS accumulation induced by LPS when compared to the control group ($p< 0.05$). In the same manner, it was observed that venlafaxine pre-treatment altered LPS-induced PRDX-3 and PRDX-5 expression in neuronal cells ($p< 0.05$). Our findings indicate the involvement of multifunctional PRDX-3 and PRDX-5 enzymes in the antioxidant effect of venlafaxine and suggest that further investigations into this pathway could provide valuable therapeutic contributions.

**Keywords:** Venlafaxine, Peroxiredoxin-3, Peroxiredoxin-5, Oxidative Stress, Antioxidant

## 1. Introduction

The uptick in the prevalence of mood disorders in recent times has resulted in an increased utilization of antidepressant drugs [1,2]. Furthermore, the need for long-term drug therapy in mood disorders has contributed to higher consumption of antidepressant drugs than other medications [3]. Despite antidepressant molecules' long history of use in treatment, their exact mechanisms of action have not yet been fully elucidated. Notably, the therapeutic effects of antidepressants differ from the monoamine hypothesis, as there is a time lag between the administration of antidepressants and the observation of clinical effects (approximately 2-4 weeks), suggesting the involvement of different molecular mechanisms [4]. Understanding the therapeutic mechanisms of antidepressants is essential for identifying new pharmacological targets.

Venlafaxine (1-[2-(diethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexane) hydrochloride, $C_{17}H_{27}NO_2$. HCl) is among the most preferred antidepressants in the serotonin-norepinephrine reuptake inhibitor (SNRI) group [1,5]. It is prescribed for managing generalized anxiety disorder, major depressive disorder, panic disorder, social anxiety disorder, and obsessive-compulsive disorder. Additionally, it is used for reducing menopause-related vasomotor symptoms, diabetic neuropathic pain, and migraine prophylaxis when other treatments are not effective [1,6,7]. Due to its broad therapeutic index and relatively faster response compared to other antidepressant molecules, venlafaxine is reported to be the second most prescribed antidepressant worldwide, following SSRIs [1,8].

The brain is vulnerable to oxidative stress (OS) due to its elevated oxygen consumption and a comparatively limited antioxidant defense system. [9]. Research has determined that changes in brain functions associated with OS, neuronal plasticity loss, and decreased frontal cortex and hippocampus volume contribute to depression [10]. It is pointed out that OS is one of the major factors attributed to these changes in brain structure and function.

Peroxiredoxin (PRDX) enzymes, which could reduce hydroperoxides and peroxynitrite formed in cells during physiological processes, play a central role [11]. PRDX enzymes (EC 1.11.1.15) are cells' most efficient peroxidase-functional antioxidant enzymes, reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides to water and alcohol [12]. Six PRDX isoforms are expressed in mammalian cells, numbered from 1 to 6, and found in different cell compartments [12,13].

PRDX enzymes regulate cell redox reactions through their peroxidase activities and act as antioxidants. Peroxides can be synthesized within cells through various pathways, such as the end products of metabolic pathways, exposure to environmental factors, or when defence cells attack other organisms. Peroxides can transform into reactive radical species within the cell, leading to OS. Moreover, it is established that peroxide signaling contributes to crucial cellular functions, including cell growth, angiogenesis, cellular senescence, programmed cell death, inflammation, and tissue healing [14]. The significance of PRDX enzymes in regulating cell homeostasis through their peroxidase effects has been established [15].

Furthermore, PRDX-1 and -2 enzymes have been shown to directly interact with transcription factors like NF-κB in both the cytosol and the nucleus, independent of their peroxidase activities. They can also influence protein expression at the gene level, leading to their characterization as multifunctional enzymes [12]. The diverse roles of these enzymes in cellular metabolism, homeostasis, defence, and cell survival have increased researchers' interest.

In literature review, both in terms of the pathogenesis of depression and the molecular mechanisms of antidepressants, the relationship between PRDX enzymes and their potent antioxidant properties, as well as their significant roles in inflammation, make it valuable to investigate the possible effects of the widely used compound venlafaxine in the treatment of depressive disorders or its potential roles in treatment mechanisms. This study aims to demonstrate, for the first time, the effects of the venlafaxine compound on the expression of PRDX-3 and PRDX-5 in neuronal cells under conditions of increased OS.

## 2. Materials and Methods

### *2.1. Cell Culture and Treatments*

The human neuroblastoma cells (SH-SY5Y, ATCC #CRL-2266™) were maintained in Dulbecco's Modification of Eagle's Medium (Biowest) containing 10% (v/v) FBS (Gibco) and 2.5 mM glutamine. Cells

were grown in an atmosphere of 5% $CO_2$ and 95% air at 37 ºC [16].

To determine the $IC_{50}$ concentration of venlafaxine in SH-SY5Y cells, they were plated at 10000 cells/ well in 96-well plates. The other day, cells were treated with various concentrations of venlafaxine HCl disolved in DMSO (0-250 µM) (Santa Cruz, #SC-201102) for 24-h.

To oxidative stress induction in SH-SY5Y, cells were treated with various concentrations of LPS dissolved in DMSO (0.1, 0.5, 1, 5 ve 10 µg/mL) (Sigma, L4391) or 1% DMSO as a control for 1-, 2-, 4- and 8-h. The findings obtained in this optimization examination reveal that, following a 4-h incubation period in SH-SY5Y cells, applying LPS at a concentration of 1 ug/mL induces a significant increase in oxidative stress levels without causing a notable change in cell viability (data were not shown).

### *2.2. Determination of Cell Viability*

Cell survival was assessed by MTT assay (Thermo Fisher Scientific, M2003) [16–18]. For experiments, cells were seeded on 6-well plates and pretreated with venlafaxine (50 or 100 µM) for 24- and 48-h. Then, the cell medium was replaced with 1 µg/ ml LPS containing fresh medium and incubated for 3-h. At the end of the treatment, cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C. Afterward, the evaluated formazon crystals were dissolved by dimethyl sulfoxide. The absorbance was measured at the wavelength of 570 nm and 650 nm using a ClarioStar plus microplate reader. Data were expressed as the % percentage of absorbance versus the control cells.

### *2.3. Determination of Intracellular ROS Production*

Intracellular reactive oxygen species levels in cells were measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay, as described previously [16]. Briefly, cells were washed once with 1x sterile PBS and then incubated in the same buffer containing 1µM of DCFH-DA (Calbiochem, Sigma, 287810) for 30 min at 37°C. A microplate reader measured the intracellular fluorescence intensity level with excitation at 488 nm and emission at 525 nm. Data were expressed as the % percentage of absorbance versus the control cells.

### *2.4. Protein Extraction and Western Blot Analysis*

After treatments, protein expression levels were analyzed by Western blot, described previously in detail [19]. Cells were lysed in lysis buffer and total protein concentration was quantified by the BCA protein assay kit (Pierce, #23225)[19,20]. Forty µg of protein were separated by 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, #4561095) and transferred onto a PVDF membrane.

The membrane was blocked with skimmed milk (5%) in PBS-T (pH 7.4), incubated with the primary antibodies (polyclonal-rabbit anti-PRDX-3, Proteintech #10664-1-AP (1:2000), and polyclonal rabbit anti-PRDX-5; Proteintech #17724-1-AP (1:1000)) for two hrs at room temperature, respectively. Afterwards, it was agitated with HRP-conjugated secondary antibodies (Pierce, 1:7500) for 1 h. Beta-actin (1:100000) was used as a loading control.

Finally, signals of the interested protein were detected using SuperSignal® West Pico (ECL, Pierce #34580) by Er Biyotek-Gen-Box CFX imaging system. Data analysis was finished by measuring the densities of immunoreactive bands via Image J software. Data analysis was finished by measuring the densities of immunoreactive bands via Image J software.

### *2.5. RNA Extraction, cDNA Synthesis and RT-PCR Analysis*

Total RNA was extracted from cells using the RNA extraction kit (Thermo Scientific, K0731) [19]. The total RNA concentration of the samples was quantified at A260/280 nm using the CLARIOstar Plus microplate reader with the LVIS plate (BMG LABTECH). A ratio of 1.95-2.00 was regarded as pure for RNA samples after measuring the absorbance260/absorbance280 ratio of the samples. cDNA Synthesis kit (Thermo Scientific, K1681) was used to reverse transcribe one microgram of total RNA using AriaMx Real-time PCR System (Agilent Technologies, USA) under the following conditions: synthesis for 30 min at 65 °C and reverse transcriptase inactivation for 5 min at 85 °C, 40 cycles, respectively.

mRNA expressions were performed using AriaMx Real-time PCR System (Agilent Technologies, USA) and PowerUp SYBR Green Master Mix (Applied Biosystems, USA). Using the software Primer3, new


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primers were created, and the sequences utilized are shown in Table 1[19]. In the assays, GAPDH was used as a housekeeping gene. The relative expression of PRDX-3 and -5 expressions was calculated as described previously [19,21]. The melting curve analysis of the PRDX-3 and PRDX-5 PCR amplicons validated their amplified specificity (data not shown). In three separate studies conducted in triplicate, the folds of changes were presented as means S.E.M.

### *2.6. Statistical Analysis*

Data was analysed using Student's t-test with SPSS 25.0 Statistical Package (IBM, SPSS Corp.). The data were presented as mean ± standard error of the mean (S.E.M.), and statistical significance was determined at a threshold of $p < 0.05$.

## 3. Results and Discussion

Figure 1 shows the effect of the venlafaxine HCl on the viability of SH-SY5Y cells and to establish the $IC_{50}$ concentration, cells were incubated with different concentrations of venlafaxine HCl (1, 5, 10, 50, 100, and 250 µM) for 24 and 48 hrs. Subsequently, the MTT assay was used to measure cell viability spectrophotometrically. The results revealed that, following a 24-hrs treatment of venlafaxine HCl, a statistically significant decrease in cell viability was observed only at a concentration of 250 µM (86.65 % ± 3.64, $p<0.05$). However, after a 48-hrs treatment, statistically significant decreases in cell viability were noted at 100 and 250 µM concentrations (86.04 ± 4.27 and 80.66 ± 4.53, respectively) ($p<0.05$). In simultaneousness with these findings, it was also determined that the $IC_{50}$ concentration of venlafaxine in SH-SY5Y cells is above 250 µM.

To investigate the effect of venlafaxine HCl against oxidative stress, cells were incubated with various doses of venlafaxine HCl (0, 1, 5, 10, 50, and 100 µM) for 24 hrs, followed by a 4-h application of LPS (1 µg/mL). Changes in intracellular ROS production were determined using the DCFH-DA method

**Table 1.** Forward and reverse primer sequences.

| Gene | Forward (5' 3') | Reverse (5' 3') | Amplicon |
|---|---|---|---|
| PRDX-3 (Gene ID:10935) | 5'-gtcgcagtctcagtggattc-3' | 5'-aacagcacaccgtagtctcg-3' | 140 bp |
| PRDX-5 (Gene ID:25824) | 5'-caagaagggtgtgctgtttg-3' | 5'-taacactcagacaggccacc-3' | 134 bp |
| GAPDH (Gene ID:2597) | 5'-agccacatcgctcagacac-3' | 5'-gcccaatacgaccaaatcc-3' | 65 bp |

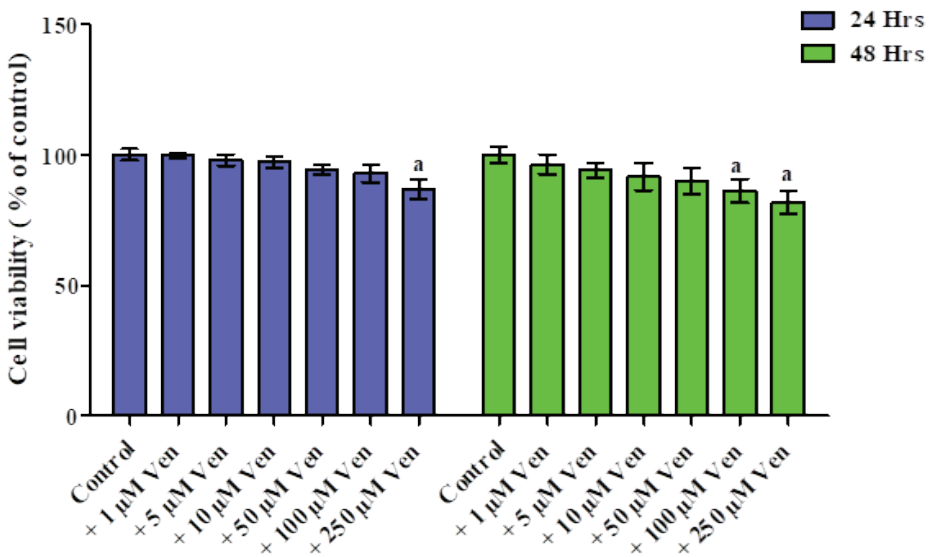


**Figure 1.** Effects of venlafaxine treatment on SH-SY5Y cell viability. [a] $p< 0.05$ *vs.* 1% DMSO-treated (control) cells. (n=3).

spectrofluorometrically. The obtained findings are presented in Figure 2 as a percentage change (mean ± S.E.M) relative to the value in cells treated with only LPS (1 µg/mL), which served as the positive control. The results shown that Venlafaxine HCl, including the lowest tested dose of 1 µM, significantly decreased the LPS-induced ROS generation in neuronal cells. The levels of intracellular ROS reduction, depending on Venlafaxine concentration, were determined as 41.53% ± 2.05, 49.91% ± 3.42, 50.17% ± 3.73, 56.76% ± 2.49, and 61.67% ± 4.06, respectively.

To examine the potential neuroprotective effect of venlafaxine HCl on oxidative stress, the cells were either incubated with only 50 and 100 µM venlafaxine for 24 hrs or treated with 1 µg/mL LPS for 4 hrs following venlafaxine pre-treatment. Cells to be used as controls were treated with DMSO, which was used to dissolve venlafaxine. After the total incubation period, intracellular PRDX-3 and PRDX-5 protein expression levels were determined using the immunoblotting method. Protein levels were normalized to β-actin as a loading control. The immunoblot images of the obtained findings are presented in Figure 3A, and the graphs representing the densitometric analysis of these findings can be found in Figure 3B.

The obtained results determined that venlafaxine HCl at concentrations of 50 and 100 µM significantly increased the expression levels of PRDX-3 proteins in SH-SY5Y cells compared to the control ($p < 0.05$). LPS (1 µg/mL) treatment did not significantly change the expression levels of PRDX-3 proteins ($p > 0.05$). However, it was observed that cells incubated with venlafaxine HCl (50 and 100 µM) for 24 hrs before LPS induction exhibited a significant decrease in PRDX-3 expression levels compared to non-treated control cells and cells treated only with LPS ($p < 0.05$).

Also, it was determined that the venlafaxine HCl at concentrations of 50 and 100 µM did not significantly change PRDX-5 protein expression levels in SH-SY5Y cells ($p > 0.05$). In contrast to the PRDX-3 findings, it was found that LPS treatment (1 µg/mL) caused a significant increase in PRDX-5 protein expression ($p < 0.05$). Furthermore, it was determined that pretreatment with venlafaxine prevented this increase in a concentration-dependent manner ($p < 0.05$).

To examine the effects of venlafaxine HCl on gene expression levels of PRDXs, we measured relative mRNA levels of PRDX-3 and PRDX-5 in SH-SY5Y cells (Figure 1). The obtained data for relative PRDX-3 and PRDX-5 mRNA levels following the application of venlafaxine at concentrations of 50 and 100 µM in SH-SY5Y cells are presented in Figure 4.

The Tm values of PRDX-3 and PRDX-5 were 78.0°C and 78.5°C, respectively (data were not shown).

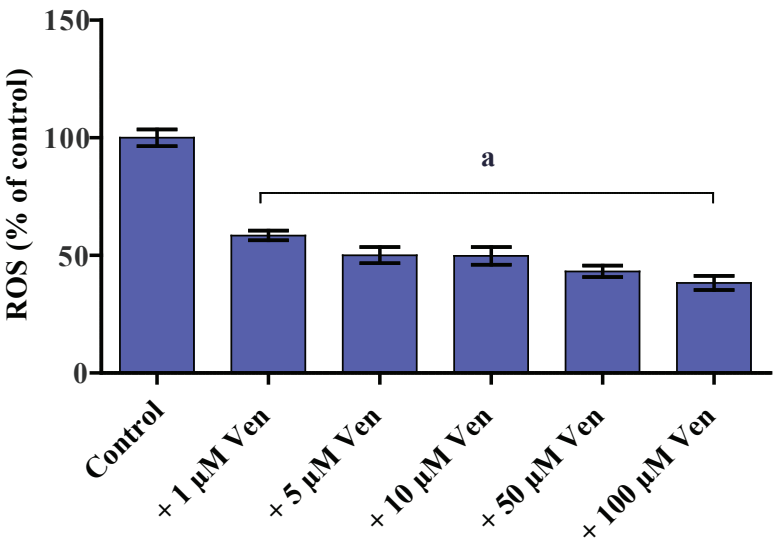


**Figure 2.** Effects of venlafaxine HCl against LPS-induced intracellular ROS accumulation in SH-SY5Y cells. [a] $p < 0.05$ *vs.* LPS-treated control cells. (n=3).


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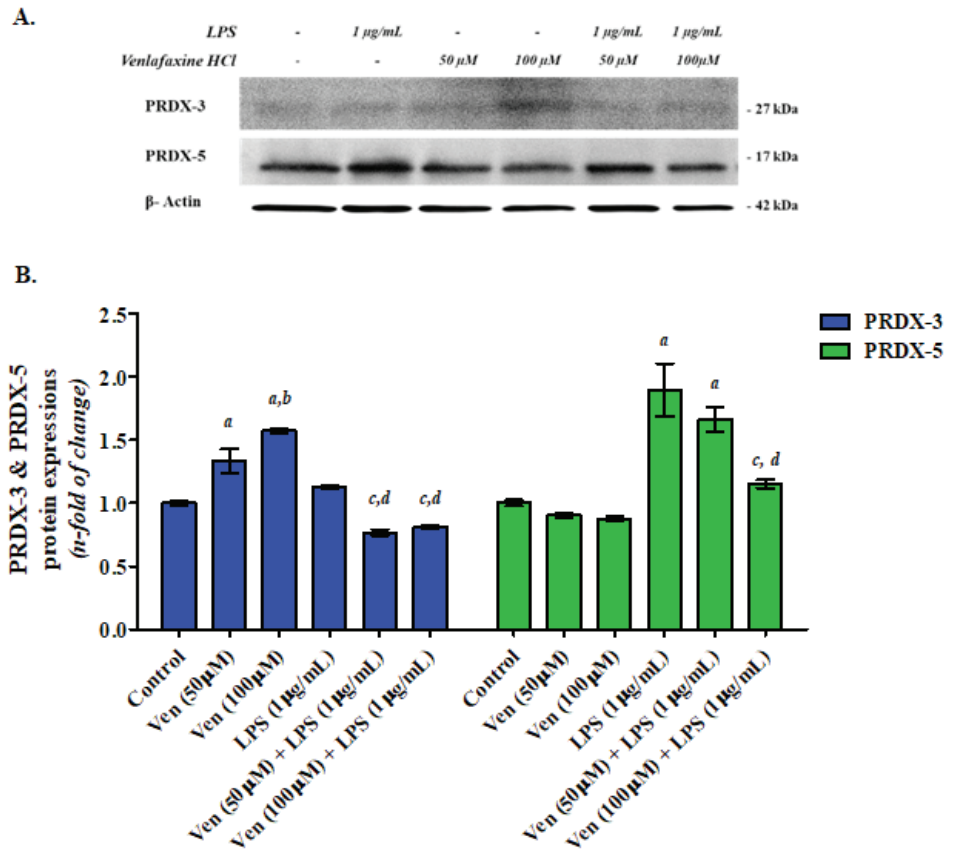


**Figure 3.** Protein expression levels of PRDX-5 and PRDX-5 in SH-SY5Y cells. [a] $p < 0.05$ vs. control; [b] $p < 0.05$ *vs.* 50 μM venlafaxine HCl-treated cells; [c] $p < 0.05$ *vs.* DMSO-treated cells; [d] $p < 0.05$ *vs.* LPS-treated cells (Ven: Venlafaxine).

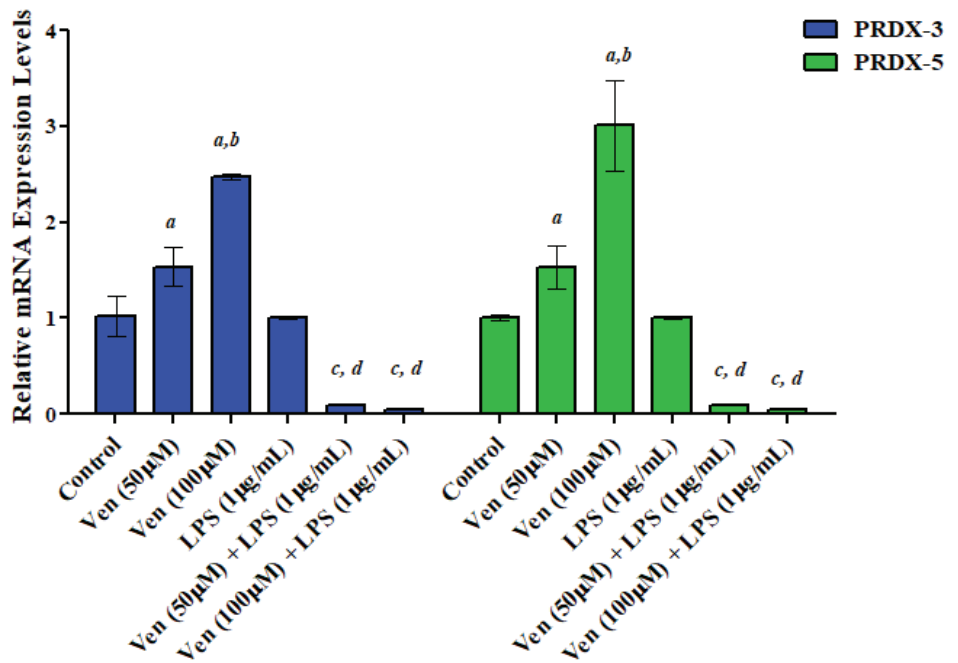


**Figure 4.** The relative mRNA expression levels of PRDX-3 and PRDX-5 enzymes in SH-SY5Y cells (n=3). [a] $p < .05$ *vs.* control; [b] $p < 0.05$ *vs.* 50 μM venlafaxine HCl-treated cells; [c] $p < 0.05$ *vs.* control; [d] $p < 0.05$ *vs.* LPS-treated cells (Ven: Venlafaxine).

It was observed that the application of Venlafaxine HCl at 50 and 100 μM concentrations in cells significantly increased PRDX-3 mRNA expression compared to the control (1.56 ± 0.29, 2.47 ± 0.22, respectively) ($p < 0.01$) (Figure 4). The application of LPS (1 μg/mL) did not lead to a significant change in PRDX-3 mRNA expression compared to the control (0.998 ± 0.08, $p > 0.01$). However, it was determined that pre-induction with Venlafaxine HCl (50 and 100 μM) before LPS induction significantly decreased PRDX-3 mRNA expression compared to the control (0.03 ± 0.01, 0.03 ± 0.01, respectively) ($p < 0.01$).

On the other hand, it was determined that the application of Venlafaxine HCl at concentrations of 50 μM and 100 μM in cells resulted in a significant increase in PRDX-5 mRNA expression compared to the control (1.53 ± 0.31, 3.01 ± 0.67, respectively) ($p < 0.01$) (Figure 4). Following the application of LPS, PRDX-5 mRNA expression levels were found to be similar to the control (1 ± 0.02) ($p > 0.01$). However, it was observed that pre-induction with 50 and 100 μM of venlafaxine HCl before LPS application significantly decreased PRDX-5 mRNA expression compared to the control (0.09 ± 0.01, 0.05 ± 0.01, respectively) ($p < 0.01$).

Numerous studies have shown that an increase in oxidative stress (OS) plays a role in the pathogenesis of many diseases affecting human health, such as neurological disorders, neuroinflammation, cancer, cardiovascular diseases, diabetes, autoimmune diseases. Therefore, research on compounds with antioxidant properties in treating diseases associated with oxidative damage is fundamental [9,22].

It has been established that OS development in neuronal cells leads to neuronal damage (neurodegeneration) by causing oxidative cell damage, neuroinflammation, and neurodegeneration [23,24]. In a study involving depression patients, it was reported that polymorphonuclear leukocytes in blood samples from these patients produced higher levels of ROS compared to healthy individuals [25]. Another investigation examining blood samples from depression patients suggested that oxidative/nitrosative stress significantly influences the disease [26]. Black *et al*., investigating the relationship between major depressive disorder and OS, showed a significant increase in oxidative DNA damage markers than the control group [9].

It has also been suggested that the effects of antidepressants on OS may play a role in their pharmacological effects, and it has been proposed that an effective strategy for depression treatment could be developed by targeting mechanisms with appropriate antioxidants.

As a result of *in vivo* and *in vitro* studies conducted to elucidate the mechanisms of action of antidepressant compounds, it has been reported that long-term administration of certain antidepressant drugs may provide protective effects against oxidative stress in brain tissue [27–29]. Although the exact mechanisms by which antidepressants act against OS have not been fully elucidated, several findings suggest that they may reduce ROS/RNS generation or enhance antioxidant defence mechanisms such as SOD or catalase. In a study conducted by Reus et al., they reported that both short and long-term administration of imipramine reduced lipid and protein oxidation in rats' prefrontal cortex and hippocampus tissues and increased the activities of SOD and catalase [30].

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, citalopram, paroxetine, fluvoxamine, or sertraline have been shown in various studies to significantly reduce NOS activity, NO, and lipid peroxidation levels with their treatment [31].

Considering that SSRIs might also play a role in the antioxidant effect of antidepressants, studies have been conducted on SNRIs. A study evaluating the effects of the adrenergic and serotonergic reuptake inhibitor mirtazapine on mRNA levels of antioxidant enzymes in U937 monocyte cells found that long-term mirtazapine treatment increased the mRNA expression levels of GST, GSH, and Cu-Zn-SOD, but short-term treatment had no effect [32]. In mice, it was found that venlafaxine treatment had a protective effect against oxidative DNA damage, reduced hippocampal MDA and NO levels, and increased GSH and total antioxidant capacity levels in the hippocampus[29]. Another study with venlafaxine showed a reduction in neuroinflammation and apoptotic cell death in the murine hippocampus [33]. The application of duloxetine, another antidepressant from the SNRI group, was reported to reduce increased ROS levels due to mitochondrial complex I inhibition [34].

A series of immunohistochemical studies were conducted on the brains of C57BL/6 mice to map the basal expression of PRDX proteins in healthy brain tissue. The findings revealed that in brain tissue, PRDX-2, -3, -4, and -5 are localized in neurons,


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while PRDX-1, PRDX-4, and PRDX-6 are found in glial neurons [35]. Information regarding the relationship between the PRDX enzyme family and the pathophysiology of depression is currently quite limited. However, the results presented from these studies are noteworthy.

In existing studies in the literature in this field, it has been reported that PRDX-1 enzyme could serve as a serum biomarker for anxiety in patients with irritable bowel syndrome and depression [36]. Another study investigating the role of PRDX-6 enzyme in psychiatric disorders showed that PRDX-6 levels decreased in the prefrontal cortex of unpredictable suicide victims and in the hippocampus of chronically mildly stressed rats [35]. This finding was supported by a recent study that demonstrated the therapeutic effect of fenofibrate through the sirtuin-PRDX-6 pathway in treating major depression [25]. However, it has also been shown that PRDX-6 expression increases in response to dexamethasone treatment in rat brains [37]. They suggested that increased PRDX-6 levels may be a response to suppress high glucocorticoid levels in depression. In a proteomic study conducted in hippocampal tissue following chronic paroxetine administration in rats, a decrease in PRDX-6 expression was observed. Another proteomic profiling study conducted on a model of chronic moderate stress resistance to escitalopram and sertraline showed increased PRDX-6 expression [27,37].

As for the relationship between the venlafaxine molecule and PRDX enzymes, only one study is available, which reported that chronic venlafaxine treatment upregulated the expression level PRDX-1 in conjunction with the anti-apoptotic BCL-2 [38]. It has been shown that venlafaxine treatment reduces hydrogen peroxide and peroxynitrite levels in mouse brain tissue and increases the expression of specific mitochondrial antioxidant genes [25,39,40].

When the findings obtained in this study are collectively evaluated, it has been determined that venlafaxine at low concentrations starting from 1 μM significantly reduces intracellular ROS levels in SH-SY5Y neuronal cells induced by LPS-induced oxidative damage and exhibits antioxidant activity. These results are supportive of the information found in the literature. Furthermore, it is demonstrated for the first time in this study that the venlafaxine HCl molecule, especially under elevated oxidative stress conditions, causes significant changes in the mRNA and protein expression levels of PRX-3 and PRDX-5.

## 4. Conclusion

This study has shown that venlafaxine HCl, an active ingredient of the frequently prescribed SNRI antidepressants in clinical practice, possesses potent antioxidant activity against oxidative stress induced by LPS in neuronal cells. Our findings, indicating the involvement of multifunctional PRX-3 and PRDX-5 enzymes in this effect, suggest that further investigations into this pathway could provide valuable therapeutic contributions. Additionally, these findings have suggested that in future studies, the interaction between venlafaxine and PRDX enzymes under various stimuli in neuroinflammation and neurodegeneration conditions could be valuable in understanding their antioxidant activities and mechanisms.

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## Conflicts of Interest

The authors state that they have no conflicts of interest to disclose.

## Statement of Contribution of Researchers

Concept – FAK; Design – FAK, MIA, AIA; Supervision – FAK; Resource – FAK; Materials -FAK; Data Collection and/or Processing – FAK, MIA, AIA; Analysis and/or Interpretation – FAK, MIA, AIA; Literature Search – FAK, MIA, AIA; Writing - FAK; Critical Reviews - FAK, MIA, AIA.

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