Exp Biomed Res 2025; 8(3): 149-160 **https://doi.org/ 10.30714/j-ebr.2025.245**

Experimental Biomedical Research *Original article*

Vortioxetine modulates Nrf2/HO-1 signaling and antioxidant defense in a cuprizone-induced demyelination model

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ABSTRACT

Aim: To evaluate the effects of vortioxetine on oxidative stress and antioxidant defense responses through the regulation of the Nrf2/HO-1 signaling pathway in a mouse model of cuprizone-induced demyelination, representing experimental Multiple Sclerosis (MS).

Method: Twenty-four male C57Bl/6 mice were randomly divided into four groups: control, cuprizone, cuprizone + vortioxetine, and vortioxetine. Demyelination was induced by administering cuprizone (10 mg/kg) via oral gavage every alternate day for a duration of 5 weeks. Vortioxetine (10 mg/kg) was delivered intraperitoneally to the appropriate groups for the same period. Biochemical analyses were conducted to assess nuclear and total Nrf2, HO-1, and total antioxidant status (TAS).

Results: Cuprizone treatment led to a significant increase in HO-1 levels compared to the control group while co-administration with vortioxetine significantly reduced HO-1 levels relative to the cuprizone group. Nuclear Nrf2 expression was elevated in the cuprizone group and significantly decreased in the vortioxetine co-treatment group. The nuclear-to-total Nrf2 ratio was significantly lower in the co-treatment group compared to cuprizone group. TAS levels were significantly increased in both the cuprizone and cuprizone + vortioxetine groups relative to control, whereas TAS values in the vortioxetine group were significantly lower when compared to the co-treatment group and the cuprizone group.

Conclusions: Vortioxetine effectively modulated the Nrf2/HO-1 pathway and reduced oxidative stress in a mouse model of demyelination. These findings suggest the potential of vortioxetine as a therapeutic agent for demyelinating diseases, such as MS.

Keywords: Heme oxygenase-1, vortioxetine, demyelination, Nrf2, cuprizone.

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Received: 2025-02-06 / Revisions: 2025-04-12

Accepted: 2025-04-23 / Published: 2025-07-01

1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) functions as a transcription factor that promotes the expression of protective proteins within cells and facilitates the synthesis of proteins involved in detoxification processes [1]. Under normal physiological conditions, its cytoplasmic concentration remains relatively low due to sequestration by Kelch-like ECH-associated protein 1 (Keap1) [2]. When oxidative stress or mitochondrial dysfunction occurs, Nrf2 separates from Keap1 and moves into the nucleus [3]. There, it triggers the synthesis of proteins such as heme oxygenase, superoxide dismutase, and detoxification enzymes by attaching to the antioxidant response element (ARE) within the nucleus [4].

The brain, which consumes the majority of oxygen in the body due to its active metabolism, is the primary organ exposed to oxidative stress [2]. Nrf2 functions as a pivotal protective factor against the persistent generation of reactive oxygen species (ROS). It is produced by neurons, oligodendrocytes, astrocytes, and microglia, and has been identified as a potential target in neurodegenerative diseases derived from oxidative stress and inflammation [5]. Studies have indicated that Nrf2 levels are notably higher in astrocytes, which aid in generating crucial antioxidants, especially in conditions like Parkinson's disease and Multiple Sclerosis [6, 7]. Studies have indicated that Nrf2 perceives low-glucose conditions as stress, prioritizes glucose metabolism in astrocytes to support cellular function during energy deprivation [8]. In diseases characterized by active inflammatory responses, such as Multiple Sclerosis, Nrf2 inhibits excessive cytokine production and neuronal death by promoting an anti-inflammatory phenotype in astrocytes [9]. Nrf2, also expressed at high levels in microglia, enhances anti-inflammatory responses [10]. In Nrf2 knockout mice, transcription factor nuclear factor-κB (NF-κB)-mediated inflammation is induced, leading to neuronal death [11]. Furthermore, Nrf2 protects neurons and glial cells by suppressing inflammasome complex formation and caspase activity [2].

Heme-oxygenase-1 (HO-1) is a protein that modulates the activity of the Nrf2 transcription factor. This inducible protein catalyzes the production of iron (Fe2+), carbon monoxide (CO), and biliverdin [2]. The by-products of HO-1, generated through the expression of the Hmox1 gene by Nrf2, exhibit anti-inflammatory properties [12, 13]. HO-1 is notably upregulated in neurodegenerative conditions such as Alzheimer's disease and provides protection by its increased expression in animal models of brain hemorrhage [14]. However, excessive HO-1 levels induced by Nrf2 can be neurotoxic, compromising neuronal viability [15, 16]. Therefore, the expression of the Nrf2/HO-1 pathway is stringently regulated in glial cells and neurons. In contrast, heme-oxygenase-2 (HO-2) is a constitutive form of the enzyme, maintaining stable expression across various tissues [17, 18]. HO-2, which is not an inducible enzyme like HO-1, is also classified as a constitutive enzyme [19]. The protective role of Nrf2/HO-1 signaling in conditions of inflammation and oxidative stress has led to the recognition of these proteins as potential pharmacological therapeutic targets [20, 21]. Various pharmacological activators of Nrf2, such as Tideglusib and Terameprocol, have been employed to demonstrate protective effects independent of the Keap1 protein, with these studies currently progressing through phase II clinical trials [22]. Vortioxetine, a multimodal antidepressant, promotes neuronal survival by enhancing antioxidant enzyme activity in Parkinson's and Alzheimer's diseases [23, 24]. This antidepressant, which diminishes the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1), acts as a serotonin reuptake inhibitor (SSRI) and is being studied for its potential effects on demyelinating and inflammatory conditions, including Multiple Sclerosis. Additionally, vortioxetine improves motor and cognitive functions by alleviating depression-like behaviors [25].

This study aimed to explore how vortioxetine affects the Nrf2/HO-1 signaling pathway in a Multiple Sclerosis experimental model. The research assessed vortioxetine's antioxidant and anti-inflammatory effects on astrocytes and microglia, along with the impact of altered HO-1 levels on cellular defense mechanisms. Furthermore, considering the potential toxicity linked to excessive HO-1 expression, the

regulation of this pathway was examined in relation to vortioxetine's effectiveness and safety. This study endeavored to elucidate the potential role of vortioxetine as a therapeutic agent in inflammatory and demyelinating diseases, such as MS.

2. Materials and methods

2.1. Experimental models

Twenty-four healthy male C57Bl/6 mice, aged 8 weeks and weighing between 19 and 21 grams, were randomly assigned to four distinct groups. The experimental protocol is depicted in Figure 1.

Control Group (a): Animals received a daily oral gavage of 1% methylcellulose solution at a dose of 10 mg/kg for five weeks. Additionally, they were administered an intraperitoneal injection of 5% 2-hydroxypropyl-β-cyclodextrin solution (vehicle used to dissolve vortioxetine) for the same duration. This group served as the experimental control and received neither cuprizone nor vortioxetine (n=6).

Cuprizone Group (b): Animals in this group were administered cuprizone dissolved in 1% methylcellulose at a dose of 400 mg/kg/day by oral gavage for five weeks. They also received intraperitoneal injections of a 5% 2-hydroxypropyl-β-cyclodextrin solution during the same period. This group represented the cuprizone-induced demyelination model (n=6).

Cuprizone + Vortioxetine Group (c): Animals in this group were administered daily oral gavage of cuprizone (400 mg/kg/day) dissolved in 1% methylcellulose for five weeks. Starting from the second week, vortioxetine was administered intraperitoneally for four weeks at a dose of 10 mg/kg (50 µl/10 g), prepared in 5% 2-hydroxypropyl-β-cyclodextrin. This study was designed to evaluate the potential therapeutic effects of vortioxetine on cuprizone-induced demyelination (n=6).

Vortioxetine Group (d): This group received a daily oral gavage of 1% methylcellulose solution for five weeks. Starting in the second week, vortioxetine was administered intraperitoneally for four weeks at a dose of 10 mg/kg (50 µl/10 g), dissolved in 5% 2-hydroxypropyl-β-cyclodextrin (n=6).

The mice were kept in a laboratory setting where the temperature was controlled between 21 and 24°C. They experienced a 12-hour cycle of light and darkness and had unlimited access to food and water. The humidity in this environment was consistently maintained between 55% and 65%.

2.2. Ethical approval

All animal procedures were conducted in accordance with the ethical standards outlined by the European Community Directive 86/609/EEC for the use of laboratory animals. Additionally, the experimental protocol received formal approval from the Ege University Regional Animal Ethics Committee (Approval No: 2024-041).

2.3. Creating a demyelinization model with cuprizone

To induce acute demyelination, 8-week-old C57Bl/6 mice were administered cuprizone (Sigma-Aldrich, St. Louis, MO, USA; Cat. No: B11231, CAS No: 370-81-0) via oral gavage for a duration of five weeks (Figure 1). The cuprizone was combined with 1% methyl cellulose (Sigma-Aldrich, St. Louis, MO, USA; Cat. No: M0512, CAS No: 9004-67-5) and vortexed to create a homogeneous suspension. Mice received a freshly prepared cuprizone solution at a dosage of 10 ml/kg daily. Cuprizone in its powder form, which does not interact with the methyl cellulose used as a solvent, was administered to both the Control group and the group receiving only Vortioxetine. The method of oral gavage administration was selected as it

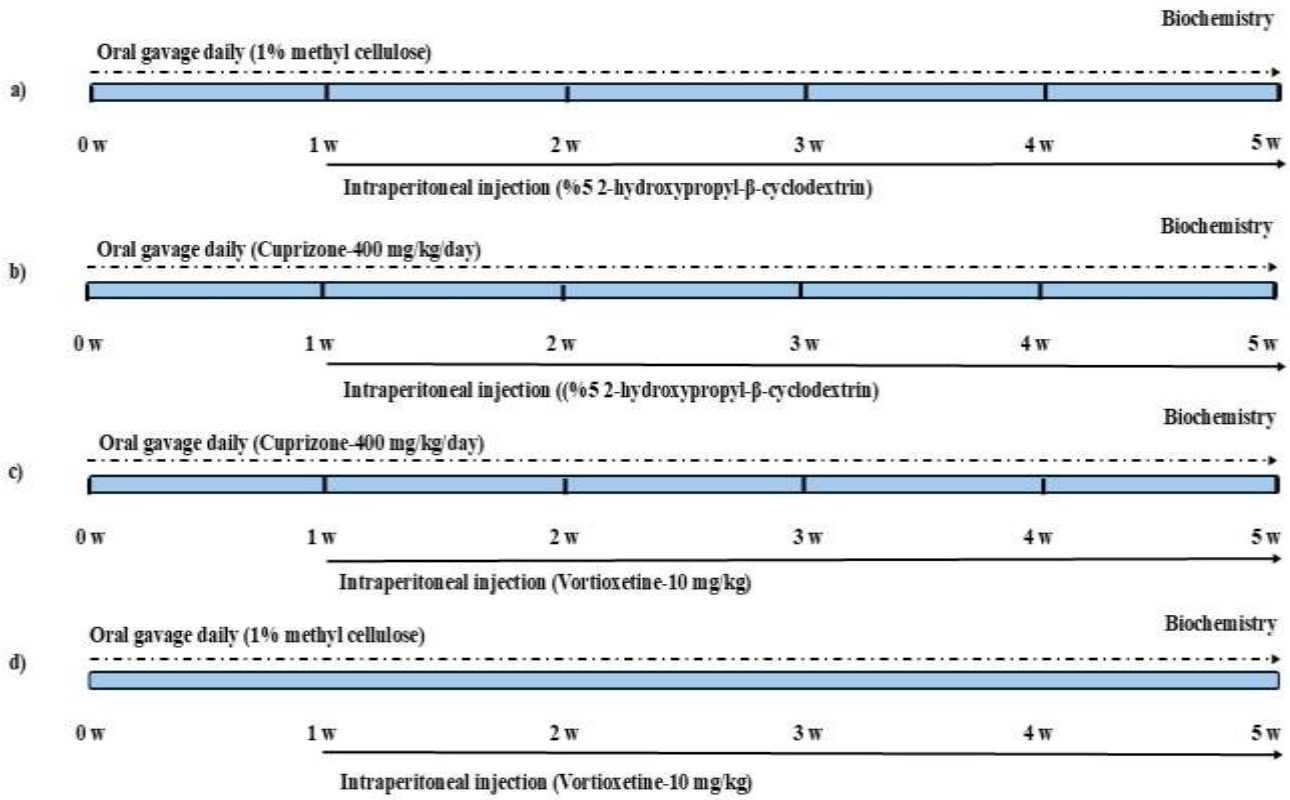

Figure 1. Experimental design (a) Control group; (b) Cuprizone group; (c) Cuprizone + Vortioxetine group; (d) Vortioxetine group.

induced maximum degeneration in the corpus callosum over the five-week period [26].

2.4. Vortioxetine administration

Vortioxetine was dissolved in 5% 2-hydroxypropyl-β-cyclodextrin (MedChemExpress, Monmouth Junction, NJ, USA; Cat. No: HY-101103, CAS No: 128446-35-5), homogenized, and subsequently administered intraperitoneally (i.p.) at a dosage of 10 mg/kg daily for a duration of five weeks to both the Cuprizone + Vortioxetine and Vortioxetine groups (Figure 1). The selected dosage of vortioxetine was informed by concentrations utilized in studies examining cognitive function and depression-like behaviors as documented in the literature. It is well-established that its solvent, 2-hydroxypropyl-β-cyclodextrin, does not exert deleterious effects on living cells and tissues, as evidenced by both *in vitro* and *in vivo* studies. For the Cuprizone and Control groups, 5% 2-hydroxypropyl-β-cyclodextrin without vortioxetine was freshly prepared and administered intraperitoneally for four weeks [25].

2.5. Biochemical analysis

Upon completion of the five-week experimental protocol, the mice were anesthetized via a high-dose intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (12 mg/kg). Following the confirmation of deep anesthesia, euthanasia was performed via cardiac perfusion using physiological saline (SF). The extracted brain tissues were then homogenized for subsequent biochemical analyses.

2.5.1. Isolation of nuclear and cytoplasmic extract

Nuclear protein extraction was performed using an ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA; Catalog No: 78833). Brain

tissues (20–100 mg) were finely minced, washed with PBS, and centrifuged at 500 × g for 5 minutes. The resulting pellet was homogenized in Cytoplasmic Extraction Reagents I and II, followed by centrifugation at 16,000 × g. The supernatant containing cytoplasmic proteins was collected. The remaining nuclear pellet was processed with Nuclear Extraction Reagent on ice, vortexed intermittently for 40 minutes, and centrifuged again at 16,000 × g for 10 minutes. The final nuclear protein fraction was stored at −80°C for subsequent analysis.

2.5.2. Nuclear factor erythroid 2-related factor 2 (Nrf2) analysis

Quantification of Nrf2 in nuclear and cytoplasmic brain extracts was performed using a mouse-specific ELISA kit (MyBioSource, Cat. No: MBS2516218). Samples and standards were loaded onto a 96-well plate and incubated at 37°C. After sequential incubations with biotinylated detection antibody and HRP-conjugated reagent, interspersed with washing steps, the colorimetric reaction was initiated using substrate solution and terminated with stop solution. Absorbance was measured at 450 nm, and Nrf2 concentrations were calculated based on the standard curve.

2.5.3. Total antioxidant status (TAS) measurement

Total antioxidant capacity in brain tissue was determined using a commercial colorimetric assay kit (Elabscience, Cat. No: E-BC-K801-M) following the manufacturer's guidelines. Briefly, tissues (~20 mg) were rinsed with PBS (0.01 M, pH 7.4), homogenized in 60% ethanol, and centrifuged at 10,000 × g for 10 minutes at 4°C. For the assay, 10 µL of sample or standard was added to the wells, followed by buffer solution. Absorbance was measured at 660 nm (A1). After the addition of the chromogenic reagent, a second absorbance reading (A2) was obtained. The difference between A2 and A1 (ΔA) was used to compute TAS values based on the standard curve.

2.5.4. Heme-oxygenase-1 (HO-1) measurement

Quantification of Heme Oxygenase-1 (HO-1) levels in mouse brain tissue was performed using a commercially available ELISA kit (Abcam, Cat. No: ab204524), following the recommended protocol. Brain tissues were homogenized in ice-cold 1X Cell Extraction Buffer PTR (prepared from supplied stock solutions). For the assay, equal volumes of tissue extract and antibody cocktail were added to pre-coated 96-well plates. After a 1-hour incubation at room temperature on a plate shaker, the wells were washed, and substrate solution (TMB) was applied. The colorimetric reaction proceeded for 10 minutes in the dark before being stopped. Absorbance was recorded at 450 nm, and HO-1 concentrations were calculated using a standard curve.

2.6. Statistical analysis

All statistical evaluations were performed using GraphPad Prism version 9. Group comparisons were assessed through one-way ANOVA, followed by Sidak's post hoc test for multiple comparisons. A p-value less than 0.05 was considered indicative of statistical significance.

3. Results

3.1. Evaluation of total and nuclear Nrf2 protein

Figure 2 presents the quantitative analysis of Nrf2 expression in the cuprizone-induced demyelination model. Nuclear Nrf2 levels were significantly elevated in the cuprizone group compared to the control ($p < 0.05$) (Figure 2a). In the group receiving vortioxetine in addition to cuprizone, nuclear Nrf2 levels were significantly reduced relative to the cuprizone-only group ($p < 0.01$).

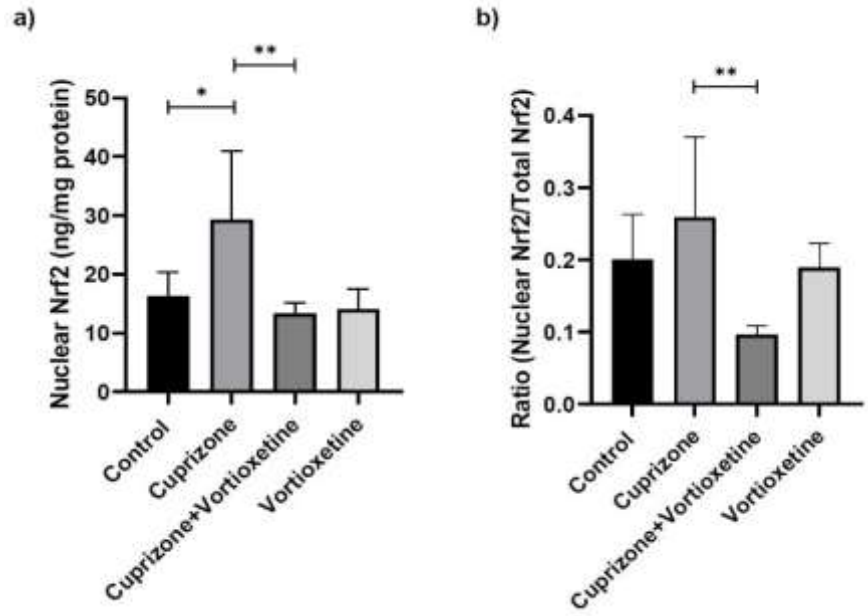

Figure 2. Nrf2 level in brain tissue. (a) Nuclear Nrf2 level in brain tissue ($F(2, 15) = 11.14$, $P < 0.01$). (b) Ratio of nuclear Nrf2 to total Nrf2 ratio ($F(2, 15) = 4.69$, $P < 0.01$). Values were presented as mean ± SEM (n = 6). *$P < 0.05$, **$P < 0.01$.

As shown in Figure 2b, the nuclear/total Nrf2 ratio did not show a statistically significant change in response to cuprizone treatment. However, a significant reduction in this ratio was observed in the cuprizone + vortioxetine group when compared to the cuprizone group ($p < 0.01$).

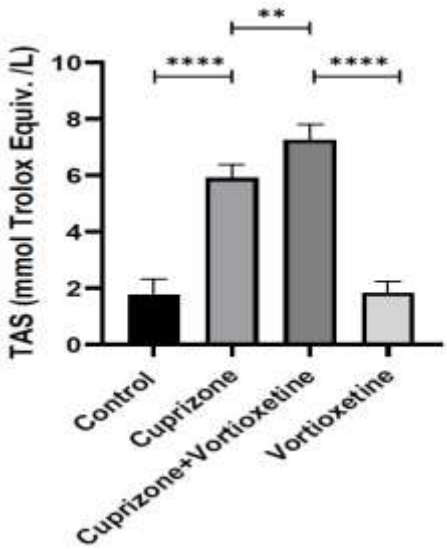

Figure 3. Total antioxidant levels in brain. Total antioxidant levels in brain ($F(2, 14) = 0.20$, $P < 0.0001$). The data were expressed as mean ± SEM (n = 6). **$P < 0.01$ ****$P < 0.0001$.

3.2. Analysis of total antioxidant level (TAS)

Figure 3 displays the total antioxidant status (TAS) levels measured in a mouse model of cuprizone-induced demyelination. The control group exhibited the lowest TAS values. TAS levels were significantly elevated in the cuprizone group ($p < 0.01$; $p < 0.0001$). The group receiving both cuprizone and vortioxetine showed the highest TAS values ($p < 0.01$), whereas the vortioxetine-only group did not differ significantly from the control group.

3.3. Evaluation of HO-1 protein amount

Figure 4 illustrates the HO-1 protein levels across experimental groups. The control group exhibited significantly lower HO-1 levels compared to all other groups ($p < 0.0001$). Cuprizone administration resulted in a significant elevation in HO-1 expression ($p < 0.0001$). In the group treated with both cuprizone and vortioxetine, HO-1 levels were significantly decreased compared to the cuprizone-only group ($p < 0.0001$), but remained significantly higher than those in the control group.

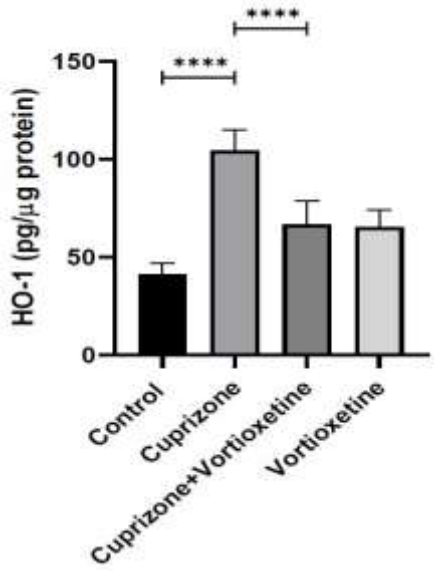

Figure 4. Hemeoxygenase-1 levels in brain. Hemeoxygenase-1 level in brain ($F(2, 14) = 0.71$, $P < 0.0001$). The data were expressed as mean ± SEM (n = 6). ****$P < 0.0001$.

4. Discussion

Sequential processes such as mitochondrial dysfunction, oxidative stress, and apoptosis significantly contribute to the pathophysiology of various neurodegenerative diseases, ultimately leading to neurological impairments. While multiple sclerosis is primarily an inflammatory condition, the presence of demyelination in the absence of axonal damage indicates that degeneration is also a critical component of its pathology [27].

Reactive oxygen species generated as a result of cellular activities activate the Nrf2 pathway, which endeavors to mitigate oxidative stress products. Antioxidant proteins, notably hemoxygenase-1, play a role in counteracting the cell's local immune response [28]. Research has indicated that the reactive oxygen species produced in multiple sclerosis (MS) cannot be maintained within physiological limits, thereby triggering neuronal damage, neuroinflammation, and demyelination [29]. The lack of active Nrf2 in the brains of individuals with Alzheimer's disease implies that Nrf2 may be inadequate for defense against MS [30]. Additionally, the presence of congenital inflammation and neuronal death in Nrf knockout mice provides evidence that Nrf2 is a vital protein in safeguarding neurons against oxidative stress [31]. Nrf2 plays a crucial role in modulating the levels of pro-inflammatory cytokines generated by oxidative stress within cells [32]. The deletion of Nrf2 in microglia, which are the brain's resident immune cells, results in the suppression of various proteins, including HO-1, a key component in defense mechanisms [33]. Dimethyl fumarate (DMF), which can oxidize the sulfhydryl (-SH) groups of Keap1, shows both antioxidative and proinflammatory effects in neurons and glial cells by stimulating the Nrf2 pathway [34, 35]. In addition, in a study, the therapeutic effect of DMF was realized by stimulating the Nrf2/HO-1 pathway in retinal ganglion cells against optic nerve damage [36]. DMF also shows a protective effect against vascular dysfunction and damage by stimulating Nrf2 [37, 38]. Recent studies have been conducted on the deterioration of vascular functions in the experimental demyelination model created with cuprizone [39].

Dimethyl fumarate, which received approval from the US Food and Drug Administration (FDA) in 2013 for the treatment of multiple sclerosis (MS), alleviates disease symptoms by promoting the nuclear translocation of Nrf2 [40]. In a cuprizone-induced MS model, an increase in neuroinflammation and oligodendrocyte apoptosis was observed in Nrf2 knockout mice [41]. In the current study, the administration of cuprizone led to an elevation in nuclear Nrf2 levels. Additionally, our findings indicate that cuprizone, which enhances oxidative stress, also activates the antioxidant system via Nrf2. In an experimental autoimmune encephalomyelitis (EAE) model, the elevation of Nrf2 levels

through DMF administration resulted in a reduction of MS symptoms and mitigated axonal degeneration [35]. Similarly, vortioxetine may exert its neuroprotective effects, at least in part, through mechanisms analogous to those of DMF, by enhancing nuclear Nrf2 expression and activating antioxidant pathways. Given recent evidence implicating vascular dysfunction in demyelination models, it can be hypothesized that vortioxetine may also improve vascular function, thereby contributing to the activation of Nrf2 and subsequent induction of cytoprotective enzymes such as HO-1. Although there is currently no direct evidence linking vortioxetine to the activation of the Nrf2/HO-1 signaling pathway or its efficacy in multiple sclerosis (MS), emerging studies on other antidepressants suggest that members of the SSRI and SNRI classes may exert secondary neuroprotective effects through antioxidant mechanisms. For instance, fluoxetine has been shown to increase Nrf2 expression and upregulate its downstream targets such as HO-1, glutamate-cysteine ligase catalytic subunit (GCLC), and NAD(P)H quinone oxidoreductase 1 (NQO1) in rodent models of corticosterone-induced depression [42]. Similarly, sertraline has been reported to enhance HO-1 expression and ameliorate doxorubicin-induced oxidative damage [43]. Among SNRIs, duloxetine was found to protect neuronal cells from oxidative injury by activating the Akt/Nrf2/HO-1 signaling cascade, while venlafaxine has been shown to modulate antioxidant gene expression and methylation status in a chronic mild stress model [44, 45]. Taken together, these findings support the notion that beyond their primary antidepressant mechanisms, certain serotonergic agents may possess secondary antioxidant or anti-inflammatory properties. Although vortioxetine is classified as a multimodal serotonergic modulator rather than a classical SSRI or SNRI, its pharmacological profile and observed effects in our study such as enhanced nuclear Nrf2 and HO-1 levels may reflect a broader class effect. These results highlight the potential of vortioxetine to modulate oxidative stress pathways, warranting further investigation into its role in neurodegenerative disorders like MS.

The molecular mechanisms by which various drugs purportedly reduce pro-inflammatory microglia and reactive astrocyte activity remain largely undefined. Vortioxetine, the focus of the present study, is a widely prescribed multimodal antidepressant. While its primary efficacy has been documented in major depressive disorders, recent investigations have explored its effects in a range of neurodegenerative diseases. In a rat model of induced Parkinson's disease, vortioxetine was found to protect dopaminergic neurons and slow the progression of both motor and non-motor symptoms by attenuating inflammation [25]. In our study, vortioxetine administration in a murine demyelination model demonstrated effects that bolster antioxidant defense mechanisms and the Nrf2 pathway. Specifically, vortioxetine may exert neuroprotective effects by elevating nuclear Nrf2 levels under oxidative stress and modulating the total/nuclear distribution of Nrf2. These findings suggest that vortioxetine may have potential applications not only in the treatment of neurological disorders but also in the prevention of conditions associated with oxidative stress. Cuprizone induces oxidative stress, thereby increasing the body's antioxidant response. However, this increase is limited and may be inadequate to manage oxidative stress effectively. Vortioxetine appears to significantly enhance antioxidant capacity, facilitating a more robust response to oxidative stress. The concurrent increase in nuclear Nrf2 levels and HO-1 stimulation may align with the mechanism by which vortioxetine augments the production

of antioxidant enzymes through activation of the Nrf2 pathway. This may represent an additional mechanism underlying the neuroprotective effects of vortioxetine. Maintaining HO-1 at an optimal level supports the balanced functioning of cellular defense mechanisms. Therefore, it can be concluded that vortioxetine not only mitigates oxidative stress but also regulates cellular responses. Our study supports the potential application of vortioxetine in managing oxidative stress via HO-1. Cuprizone induces oxidative stress, thereby augmenting the body's antioxidant response. However, this augmentation is limited and may be inadequate for managing oxidative stress. In contrast, vortioxetine appears to significantly enhance antioxidant capacity, facilitating a more effective response to oxidative stress. The concurrent increase in nuclear Nrf2 levels and HO-1 stimulation may align with the mechanism by which vortioxetine enhances the production of antioxidant enzymes through activation of the Nrf2 pathway. This may represent an additional mechanism underlying the neuroprotective effects of vortioxetine. Maintaining HO-1 at an optimal level supports the balanced functioning of cellular defense mechanisms. Consequently, it can be concluded that vortioxetine not only mitigates oxidative stress but also regulates cellular responses. Our study supports the potential application of vortioxetine in managing oxidative stress via HO-1. This study elucidates the neuroprotective effects of vortioxetine and its potential therapeutic application in pathologies associated with oxidative stress. Our findings demonstrate that vortioxetine offers a robust defense mechanism against oxidative stress through the activation of the Nrf2/HO-1 pathway, which may play a crucial role in neurodegenerative processes such as demyelination. Although the effects of vortioxetine on neuroinflammation and oxidative stress have been limited, our study addresses this gap and provides a novel perspective on the drug's mechanistic effects. These findings suggest that vortioxetine may serve as a novel treatment strategy not only for neurological diseases but also for other disorders associated with oxidative stress. Future studies investigating the effects of vortioxetine in various neurodegenerative models and elucidating the drug's effects on oxidative stress, energy metabolism, inflammation, and cellular integrity may significantly contribute to the literature and clinical practice. Consequently, vortioxetine may represent an efficacious pharmacological agent for neurodegenerative diseases.

Acknowledgement: *The authors express their gratitude to the staff of the Ege University Experimental Animals Unit for their dedicated support of the laboratory studies.*

Funding: *The authors received no financial support for the research, authorship, and/or publication of this article.*

Conflict of Interest: *The authors declared no conflict of interest.*

Ethical Statement: *The animal protocols followed the European Community Directive (86/609 ECC) on the care and use of laboratory animals, as well as the Regional Animal Care Committee (Ege University, Approval number. 2024-041).*

Informed Consent

Patient approval has not been obtained as it is performed on animals.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Open Access Statement

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