ORIGINAL ARTICLE

Curcumin-piperine Therapy Enhances Neuroprotection in the Brainstem of a Cuprizone-induced Multiple Sclerosis Model

Mohammad Zubair Alam[1,2], Muhammad Yasir[1,3], Mohammad Imran Khan[4,5]

[1]Neuroscience and Geroscience Research Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia, [2]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia, [3]Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia, [4]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, [5]Research Centre, King Faisal Specialist Hospital and Research Centre, Jeddah, Saudi Arabia

Abstract

Introduction: Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system characterized by neuroinflammation, oxidative stress, and demyelination. The cuprizone (CPZ) model is widely used to study these pathological mechanisms. This study aimed to evaluate the neurotoxic effects of CPZ on the brainstem and investigate the neuroprotective efficacy of curcumin and piperine nanoformulations prepared in black seed oil. **Materials and Methods:** Seventy-five male Swiss albino mice were assigned to five groups: Control, CPZ, CPZ with blank formulation, CPZ with curcumin formulation, and CPZ with curcumin-piperine formulation. Mice received CPZ for 5 weeks to induce demyelination, followed by 2 weeks of treatment. Biochemical analyses assessed oxidative stress markers (catalase, superoxide dismutase [SOD], malondialdehyde [MDA]), inflammatory markers (tumor necrosis factor-alpha, interferon-gamma, glial fibrillary acidic protein, macrophage inflammatory protein-1, C-C motif chemokine ligand-5), and myelin integrity (myelin basic protein [MBP]). **Results:** Results showed that CPZ significantly decreased antioxidant enzyme activity, increased MDA levels, and elevated inflammatory markers. Treatment with curcumin-piperine nanoformulation significantly restored catalase and SOD activity, reduced MDA and inflammatory markers, and improved MBP levels, suggesting enhanced remyelination. **Discussion:** The curcumin-piperine combination demonstrated superior neuroprotective and anti-inflammatory effects compared to curcumin alone. **Conclusion:** These findings highlight the potential of curcumin-piperine nanoformulations in mitigating MS-related neurodegeneration and suggest their promise for future therapeutic applications.

Key words: Black seed oil, cuprizone, curcumin, demyelination, multiple sclerosis, neuroinflammation, oxidative stress, piperine

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS) characterized by demyelination, axonal damage, and neurodegeneration. It is one of the most common causes of neurological disability in young adults, affecting over 2.8 million people worldwide.[1] The pathogenesis of MS involves a complex interplay between genetic predisposition, environmental factors, and immune dysregulation, leading to inflammation, oxidative stress, and subsequent damage to myelin sheaths and neurons. Neuroinflammation, a hallmark of MS, is driven by the activation of microglia and astrocytes, infiltration of immune cells, and the release of pro-inflammatory cytokines and chemokines. These processes

Address for correspondence:
Mohammad Zubair Alam, Neuroscience and Geroscience Research Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.
E-mail: mohdzubairalam@yahoo.com

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disrupt the blood–brain barrier, exacerbate neuronal damage, and impair remyelination, contributing to the progressive nature of the disease.[2]

Neuroinflammation plays a pivotal role in the initiation and progression of MS. Activated microglia and astrocytes release pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon-gamma (IFN-γ), which perpetuate the inflammatory cascade and contribute to demyelination and axonal loss.[3] In addition, chemokines, such as C-C motif chemokine ligand 5 (CCL-5) and macrophage inflammatory protein-1 (MIP-1) recruit immune cells to the CNS, further amplifying the inflammatory response. The resulting oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, exacerbates neuronal damage and impairs repair mechanisms.[4] Understanding the molecular mechanisms underlying neuroinflammation and oxidative stress is crucial for developing effective therapeutic strategies for MS.

The cuprizone (CPZ) model is a widely used experimental model to study demyelination and remyelination in MS. CPZ, a copper chelator, induces toxic injury to oligodendrocytes, the myelin-producing cells of the CNS, leading to demyelination and neuroinflammation.[5] This model mimics key features of MS, including microglial activation, astrogliosis, and the release of pro-inflammatory cytokines. The CPZ model is particularly valuable for studying the mechanisms of demyelination and testing potential therapeutic interventions. However, unlike the autoimmune-driven pathology of MS, CPZ-induced demyelination is primarily a result of direct oligodendrocyte toxicity, making it a useful tool for investigating non-immune-mediated mechanisms of neurodegeneration.[6]

Oxidative stress is a critical contributor to the pathogenesis of MS and other neurodegenerative diseases. In MS, the excessive production of ROS by activated microglia and infiltrating immune cells overwhelms the antioxidant defense mechanisms, leading to lipid peroxidation, protein oxidation, and DNA damage.[7] These oxidative modifications disrupt cellular homeostasis, impair mitochondrial function, and trigger apoptotic pathways, ultimately resulting in neuronal and oligodendrocyte death. Moreover, oxidative stress exacerbates neuroinflammation by activating nuclear factor-kappa B (NF-κB) and other pro-inflammatory signaling pathways, creating a vicious cycle of inflammation and oxidative damage.[8] Targeting oxidative stress and inflammation represents a promising therapeutic approach for mitigating neurodegeneration in MS.

Inflammatory markers, such as TNF-α, IFN-γ, glial fibrillary acidic protein (GFAP), MIP-1, and CCL-5 are critical indicators of neuroinflammation and disease progression in MS and the CPZ model. TNF-α, a key pro-inflammatory cytokine, is elevated in the CNS of MS patients and CPZ-treated animals, contributing to demyelination and axonal damage. IFN-γ, another pro-inflammatory cytokine, exacerbates neuroinflammation by activating microglia and promoting the release of additional inflammatory mediators. GFAP, a marker of astrocyte activation, is upregulated in response to CNS injury and serves as a biomarker of neuroinflammation.[9] Chemokines, such as MIP-1 and CCL-5 play a crucial role in recruiting immune cells to the CNS, amplifying the inflammatory response and contributing to tissue damage.[10] Monitoring these markers provides valuable insights into the mechanisms of neuroinflammation and the efficacy of therapeutic interventions.

Curcumin, the active compound derived from the turmeric plant Curcuma longa, has garnered significant attention for its potent anti-inflammatory, antioxidant, and neuroprotective properties. Curcumin inhibits the activation of NF-κB and other pro-inflammatory signaling pathways, reducing the production of cytokines, such as TNF-α, IL-1β, and IFN-γ. In addition, curcumin scavenges ROS, enhances antioxidant enzyme activity, and protects against oxidative stress-induced neuronal damage.[11] However, the therapeutic potential of curcumin is limited by its poor bioavailability due to low solubility, rapid metabolism, and systemic elimination. Piperine, a bioactive alkaloid found in black pepper *Piper nigrum*, has been shown to enhance the bioavailability of curcumin by inhibiting its metabolism and increasing its absorption.[12] The combination of curcumin and piperine has demonstrated synergistic anti-inflammatory and neuroprotective effects, making it a promising therapeutic strategy for MS and other neurodegenerative diseases.

Black seed, *Nigella sativa,* and its oil have been used for centuries in traditional medicine for their wide-ranging health benefits. Black seed oil (BSO) is rich in bioactive compounds, including thymoquinone, which exhibits potent anti-inflammatory, antioxidant, and immunomodulatory properties.[13] Thymoquinone inhibits the production of pro-inflammatory cytokines, reduces oxidative stress, and protects against neuronal damage in experimental models of neuroinflammation and neurodegeneration.[14] In addition, BSO has been shown to enhance the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase, further mitigating oxidative stress. These properties make BSO a promising adjunct therapy for MS and other inflammatory disorders.

This study aimed to evaluate the neurotoxic effects of CPZ on the brainstem region of the mouse brain, focusing on its ability to induce demyelination, oxidative stress, and neuroinflammation. This included assessing changes in key antioxidant enzymes/proteins and inflammatory markers. The study sought to determine the neuroprotective efficacy of various curcumin and piperine nanoformulations prepared in BSO. This involved examining their ability to mitigate CPZ-induced oxidative stress, inflammation, and demyelination in the brainstem.

MATERIALS AND METHODS

Animals

Seventy-five Swiss albino male mice (SWR/J) weighing between 21 and 25 g were acquired from the King Fahd Medical Research Center (KFMRC) animal housing unit at King Abdulaziz University in Jeddah, Saudi Arabia. A 12-h light/dark cycle, with the light cycle occurring between 7:00 am and 7:00 pm, was implemented to maintain mice at a suitable room temperature of 23 ± 2°C and a humidity level of 65%. Food and water were freely available to every mouse. Animal studies were carried out in compliance with KFMRC's animal unit committee rules. The biomedical ethics research committee at King Abdulaziz University authorized the study protocol (approval No. ACUC-22-1-2 dated April 13, 2022), which complied with the guidelines established by the KFMRC's Animal Care and Use Committee. The research adhered to the "System of Ethics." The study was authorized by Royal Decree No. M/59 dated August 24, 2010, and it conformed with the "System of Ethics of Research on Living Creatures" rules created by King Abdulaziz City for Science and Technology. From June 01, 2022, to July 16, 2022, the study was conducted at the King Fahad Medical Research Center in Jeddah, Saudi Arabia.

CPZ and nanoformulations

CPZ (C9012-25G) was obtained from Sigma-Aldrich (Bangalore, India). Acute demyelination in mice was induced by feeding them 0.2% w/w CPZ mixed rodent chow for 5 weeks as described earlier.[15] A nanoformulation of curcumin with and without piperine was prepared by dissolving it in BSO as described and characterized earlier.[16] Briefly, different ratios of surfactant, cosurfactant, and/or cosolvent were added to the BSO to prepare these nanoformulations. A set quantity of cosurfactant (I988) and surfactant (CRH40) was used in the formulation of BSO to investigate its impact on formulation performance. The resultant liquid, which is essentially a self-nanoemulsifying drug delivery system (SNEDDS), was homogenized and kept until needed in an airtight 3 mL glass vial. The SNEDDS has a solubility of 38.4 mg/g for curcumin and 45.0 mg/g for piperine. The droplet size of drug-free SNEDDS was 27.94 nm, whereas the droplet size of curcumin and piperine loaded SNEDDS was 51.18 nm. The zeta potential of drug-free SNEDDS (−23.8 mV) and curcumin-piperine loaded SNEDDS (−19.3 mV) indicated a good physical stability of the resultant emulsion. The zeta potential value did not change significantly after curcumin-piperine loading. Curcumin was administered at 10 mg/kg, a dosage based on previous research.[17] The blank and curcumin-piperine nanoformulations were diluted in normal saline to achieve concentrations of 3 mg/kg piperine and 10 mg/kg curcumin per 0.1 mL for each mouse. Daily intraperitoneal injections of 0.1 mL of the formulation were administered to each mouse between 11:00 a.m. and 1:00 p.m. Dosages were adjusted weekly based on the mice's weight measurements.

Experimental design

The study lasted for 7 weeks in total, in which CPZ was administered mixed with rodent chow for the first 5 weeks to all groups except the control. After week 5, the CPZ was removed from the diet and lasted for the next 2 weeks (weeks 6 and 7). A total of 15 mice, each group was randomly assigned to five primary groups.[1] For 7 weeks, the control (CTN) group was given standard chow and 0.1 mL of saline intraperitoneally. The second group (CPZ) was given CPZ-mixed chow for 5 weeks and 0.1 mL of normal saline intraperitoneally. The third group received 0.1 mL of BSO-based blank formulation administered intraperitoneally along with CPZ-mixed chow daily for 5 weeks. The fourth group was given CPZ-mixed chow and a BSO-based formulation of curcumin (0.1 mL) intraperitoneally for 5 weeks. The fifth group was given CPZ-mixed chow and a BSO-based nanoformulation of curcumin with piperine for 5 weeks. CPZ feeding was discontinued in all groups at the conclusion of the week 5 (CPZ toxicity stage), but treatment with various nanoformulations continued until the end of week 7. Group names and designations: (1) CTN group; (2) CPZ group; (3) blank/vehicle formulation of BSO (BFB) group; (4) nanoformulation of curcumin in BSO (CFB) group; and (5) curcumin-piperine formulation in BSO (CPB) group.

Sample collection

By the conclusion of the 5th week, a random selection of 7 mice from each group was used to induce isoflurane anesthesia and decapitate them to end their lives. The whole brain of each mouse was then obtained by dissecting it. To get certain brain areas, including the hippocampus, frontal cortex, cerebral cortex, brainstem, and cerebellum, the obtained brains were further dissected. These brain slices were kept for future research at −80°C after being immersed in RNA-later solution. At the conclusion of the study (week 7), the remaining mice underwent the same process.

Biochemical analysis

Tissues were homogenized in radioimmunoprecipitation assay lysis buffer (R-0278; Sigma) containing phenylmethylsulfonyl fluoride at the prescribed doses, Halt™ phosphatase inhibitor (Thermo-Fisher Scientific), and protease inhibitor cocktail (cOmplete™, Roche) for use in various biochemical assays. After centrifuging the homogenate at 15000 × g for 20 min, the supernatant was separated and stored for further biochemical examination. With the use of a colorimetric assay kit obtained from SolarBio (Beijing, China), the SOD, CAT, acetylcholinesterase (AChE), and malondialdehyde (MDA) levels were determined. Enzyme-linked immunosorbent assay kits (colorimetric) from

Sunlong Biotech Co., Ltd. (Hangzhou, China) were used to measure the levels of CD4, CD8, TNF-α, GFAP, INF-γ, MIP-1, and CCL-5 in accordance with the manufacturer's instructions.

Statistical analysis

The changes and comparisons between different parameters at the end of the toxicity stage (week 5) and healing stage (week 7) were statistically analyzed using One-way analysis of variance (ANOVA) followed by Tukey's analysis. Alterations in the results were considered significant when the $P \leq 0.05$. Most of the statistical analyses were performed using Microsoft Excel and Social Science Statistics freely available at https://www.socscistatistics.com/.

RESULTS AND DISCUSSION

Effects on antioxidant enzyme activity

At the end of the demyelination phase (week 5), the CPZ group exhibited the lowest catalase activity (6.17 ± 1.11 U/mg protein), significantly lower than the CTN group (19.83 ± 1.78 U/mg protein, $P \leq 0.001$) [Table 1]. This decline suggests that CPZ-induced oxidative stress severely impairs antioxidant defense mechanisms in brainstem, in agreement with previous findings that CPZ administration depletes antioxidant enzyme activity.[18] The BFB, CFB, and CPB treatment groups demonstrated intermediate catalase levels, with CPB (16.59 ± 1.74 U/mg protein) exhibiting the highest activity among the treated groups, indicating that the curcumin-piperine combination may enhance antioxidant defense. By week 7, catalase activity in the CPZ group increased by 50.24%, likely due to the removal of CPZ exposure, supporting the notion that oxidative stress is reversible to some extent upon cessation of the toxicant.[19] The CPB group maintained the highest catalase levels, underscoring its potential neuroprotective effects.

Regarding SOD activity, the CPZ group had the lowest levels at week 5 (11.61 ± 1.34 U/mg protein), while the CNT group had the highest (21.28 ± 2.29 U/mg protein). CPB (19.76 ± 2.48 U/mg protein) showed the highest SOD levels among treatment groups, indicating its potential in mitigating oxidative damage [Table 1]. By week 7, SOD levels in the CPZ group increased by 33.07%, suggesting a recovery in antioxidant capacity. The slight decrease in SOD levels in the CNT group may be attributed to natural aging-related oxidative stress.[20] The CPB group consistently maintained high levels of SOD, reinforcing its effectiveness in oxidative stress regulation.

Effects of CPZ and other treatments on CD4 and CD8 protein

CD4 and CD8 are well-established markers of immune activation and inflammation. In this study, at week 5, the CPZ group exhibited significantly elevated CD4 levels (5271.30 ± 571.34 pg/mg protein), which were markedly higher than the CTN group ($P \leq 0.001$). This increase indicates a robust inflammatory response in brainstem triggered by CPZ-induced toxicity, which is known to induce both demyelination and immune system activation.[21] In contrast, treatment groups, particularly the CPB group, showed a significant reduction in CD4 levels, suggesting that these treatments effectively modulate immune responses and reduce inflammation [Table 1]. By week 7, the CPZ group demonstrated a 10.76% decrease in CD4 levels. This decrease in CD4 levels observed in the CPZ group after CPZ withdrawal suggests that inflammation begins to subside after the cessation of CPZ treatment. However, the failure to return to baseline levels indicates that the immune response does not fully resolve, which is consistent with findings from other studies suggesting that CPZ-induced demyelination can have lasting effects on the immune system and brain pathology.[22]

A similar trend was observed for CD8 levels [Table 1]. At week 5, the CPZ group exhibited significantly higher CD8 levels compared to the CNT group (9.12 ± 1.33 ng/mg protein versus 1.47 ± 0.39 ng/mg protein, $P \leq 0.0001$), further supporting the presence of an inflammatory response. The CPB treatment group again demonstrated significantly lower CD8 levels, reinforcing its potential as an anti-inflammatory agent. Following CPZ withdrawal, the CPZ group experienced a 17.65% reduction in CD8 levels, which suggests partial immune recovery. On the other hand, the CNT group exhibited a notable 40.81% increase in CD8 levels by week 7. This increase could be attributed to age-related immune alterations, as older individuals often experience an increase in immune system activity, particularly among T cells, as part of a compensatory response.[23]

The elevated levels of CD4 and CD8 in the CPZ group at week 5 strongly suggest an inflammatory response associated with CPZ-induced demyelination, which has been consistently shown to activate the immune system, particularly T cells, in both animal models and human studies.[21] CPZ toxicity is known to initiate a cascade of immune responses, including increased T-cell activation, which may contribute to the pathogenesis of neuroinflammatory diseases, such as MS. The marked reduction in CD4 and CD8 levels in the CPB group supports the notion that this treatment possesses anti-inflammatory properties, possibly by modulating T-cell activity or reducing inflammatory cytokine production.

Effects of CPZ and other treatments on AcetylcholinesteraseAChE levels

Acetylcholinesterase (AChE) is a critical enzyme in the process of neurotransmission, playing a vital role in the breakdown of acetylcholine at synaptic junctions, thus regulating neuronal communication.[24] In this study, the AChE activity in the CPZ group was significantly reduced at week 5 [Figure 1], with an observed activity of 12.34 ± 1.44 U/mg protein, compared to the CTN group, which displayed

Table 1: Levels of Catalase, SOD, CD4 and CD8 in brainstem at the end of week 5 and week 7

Group	Catalase (U/mg protein)			SOD (U/mg protein)		
	Week 5	Week 7	Percentage change	Week 5	Week 7	Percentage change
CNT	19.83±1.78	15.36±1.44	22.54↓	21.28±2.29	20.16±1.42	5.26↓
CPZ	6.17±1.11	9.27±0.97	50.24↑	11.61±1.34	15.45±1.69	33.07↑
BFB	11.34±1.33	12.76±0.81	12.52↑	13.65±1.65	16.19±2.11	18.60↑
CFB	13.47±1.08	14.94±1.59	10.91↑	18.85±2.35	19.69±2.07	4.45↑
CPB	16.59±1.74	17.28±2.17	4.16↑	19.76±2.48	20.67±1.94	4.61↑
P-value	***CNT×CPZ **CNT×BFB **CPB×CPZ *CNT×CFB, CPB *CPB×BFB, CFB *CPZ×BFB, CFB NS=CFB×BFB	*CNT×CPZ **CPZ×CPB *CPZ×BFB, CFB NS=CNT×BFB, CFB, CPB		**CNT×CPZ, BFB **CPB×CPZ **CFB×CPZ NS=CNT×CFB, CPB NS=CFB×CPB	*CNT×CPZ, BFB *CPB×CPZ, BFB *CFB×CPZ, BFB NS=CNT×CPB, CFB NS=CPZ×BFB	
Group	**CD4 (pg/mg protein)**			**CD8 (ng/mg protein)**		
	Week 5	**Week 7**	**Percentage change**	**Week 5**	**Week 7**	**Percentage change**
CNT	2146.61±207.59	2228.03±298.74	3.79↑	1.47±0.39	2.07±0.43	40.81↑
CPZ	5271.30±571.34	4703.65±524.36	10.76↓	9.12±1.33	7.51±0.76	17.65↓
BFB	4162.07±384.15	3681.67±436.51	11.54↓	6.88±0.81	5.37±0.32	21.95↓
CFB	2709.34±190.38	2257.51±168.45	16.67↓	3.57±0.45	4.12±0.56	15.41↑
CPB	2587.13±268.78	2146.25±247.18	17.04↓	2.77±0.52	3.30±0.41	19.13↑
P-value	***CNT×CPZ **CNT×BFB *CNT×CFB, CPB ***CPB×CPZ ***CFB×CPZ *BFB×CPZ NS=CFB×CPB	***CNT×CPZ **CNT×BFB NS=CNT×CFB, CPB **CPZ×CFB, CPB *CPZ×BFB		****CNT×CPZ, BFB ****CPZ×CPB *CFB×CPB ***CPZ×CFB *BFB×CPZ	****CNT×CPZ ***CNT×BFB **CNT×CFB *CPZ×BFB *CNT×CPB *CFB×BFB NS=CFB×CPB	

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$; NS: Non-significant. One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. SOD: Superoxide dismutase, CNT: Control, CPZ: Cuprizone, BFB: Blank/vehicle formulation of black seed oil, CFB: Nanoformulation of curcumin in black seed oil, CPB: Curcumin-piperine formulation in black seed oil ↓ symbol means percent decrease in the amount of protein at Week 7 in comparison to the amount of same protein at Week 5. ↑ symbol means percent increase in the amount of protein at Week 7 in comparison to the amount of same protein at Week 5

a higher activity level of 30.16 ± 3.17 U/mg protein. This decrease in AChE activity in brainstem suggests neurotoxicity induced by CPZ exposure, a known model for demyelination. Treatment with compounds, such as CPB and CFB, which have known neuroprotective properties, led to a significant increase in AChE activity relative to the CPZ group. This increase can be attributed to the neuroprotective effects of these treatments, which may have mitigated the neurotoxic impact of CPZ on neurotransmission. Upon CPZ withdrawal, a 22.26% increase in AChE activity was observed in the CPZ group at week 7, although the activity levels remained lower than those of the CNT group [Figure 1]. This suggests that while some recovery occurred after CPZ withdrawal, the neurotoxic effects were not fully reversed, indicating incomplete recovery of neurotransmission function. The CPB and CFB groups displayed a minor decline in AChE activity after CPZ withdrawal, but their levels still remained significantly higher than those of the CNT group, indicating that the neuroprotective effect persisted, albeit with some degree of stabilization over time. The observed reduction in AChE activity following CPZ administration supports the idea that CPZ induces neurotoxicity, particularly by disrupting cholinergic neurotransmission, a hallmark of neurodegenerative processes.[24] The increased AChE activity in the CPB and CFB groups suggests that these treatments may protect against CPZ-induced neurotoxicity, possibly through anti-inflammatory or neurotrophic mechanisms, which have been previously linked to neuroprotective therapies in experimental models of demyelination.[25]

Effects of CPZ and other treatments on lipid peroxidation

MDA levels, a widely recognized marker of lipid peroxidation, were significantly elevated in the CPZ group

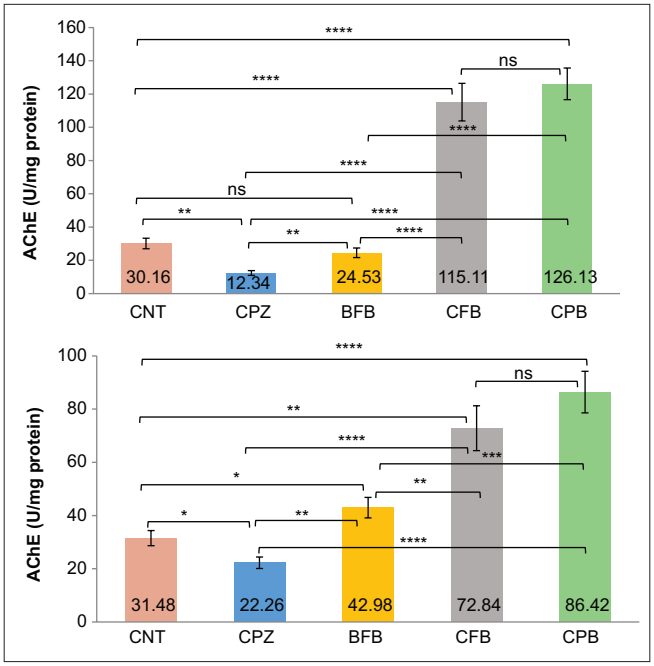

Figure 1: Level of acetylcholinesterase in the brainstem at the end of the demyelination stage (week 5) and at the end of the remyelination stage (week 7). One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$; NS: Non-significant

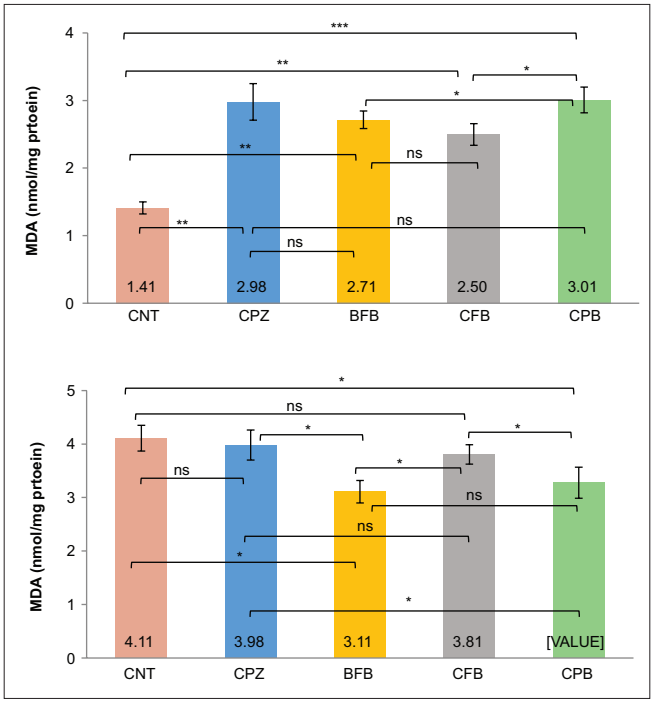

Figure 2: Level of malondialdehyde in the brainstem at the end of the demyelination stage (week 5) and at the end of the remyelination stage (week 7). One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$; NS: Non-significant

at week 5 [Figure 2], indicating heightened oxidative stress as a consequence of CPZ-induced demyelination.[26] This increase in MDA reflects the damage to cellular membranes and other lipids resulting from the production of ROS, which is commonly observed in neuroinflammatory and neurodegenerative conditions. Despite the administration of treatment in the BFB, CFB, and CPB groups, MDA levels remained comparable to the CPZ group at this time point, suggesting that the treatments did not significantly reduce lipid peroxidation during this phase of the study [Figure 2]. However, by week 7, MDA levels in the CNT group exhibited a slight increase, which may be attributed to the natural process of aging-related lipid peroxidation, as oxidative stress tends to accumulate with age. In contrast, although MDA levels in the CPZ group remained elevated, indicating sustained oxidative damage, the treatment groups showed some mitigation of oxidative stress, suggesting a potential protective effect of these treatments, albeit not sufficient to completely reverse CPZ-induced damage.

The elevated MDA levels are consistent with the understanding that CPZ-induced demyelination triggers a cascade of ROS production, which leads to lipid peroxidation and subsequent neuronal injury.[26] This oxidative stress is not only a direct consequence of CPZ exposure but also a factor that exacerbates the inflammatory response and impedes neuroprotection, making it a critical target for potential therapeutic interventions. Interestingly, the treatments in the BFB, CFB, and CPB groups did not significantly reduce MDA levels at week 5, suggesting that while these compounds may have anti-inflammatory or neuroprotective properties, their effects on lipid peroxidation stress might take longer to manifest. This observation aligns with previous studies, which have shown that the beneficial effects of neuroprotective agents often become more apparent in later stages of recovery or after prolonged treatment.[27]

At week 7, the slight increase in MDA levels in the CTN group could be due to age-related increases in oxidative stress, as lipid peroxidation tends to accumulate with age [Figure 2], further compounding the challenges of managing neurodegenerative diseases.[28] The persistence of elevated MDA levels in the CPZ group after CPZ withdrawal highlights the long-lasting effects of CPZ toxicity, which can result in prolonged oxidative damage and hinder full recovery. This finding is consistent with the literature, which suggests that while CPZ-induced demyelination is partially reversible, oxidative stress may persist even after the cessation of CPZ treatment, potentially contributing to long-term neuronal dysfunction and impairment.[29] The partial reduction in oxidative stress observed in the treatment groups (BFB, CFB, and CPB) suggests that these compounds may offer some level of protection against CPZ-induced oxidative damage. However, their effects may not be sufficient to completely reverse the damage caused by CPZ, emphasizing the need for

ongoing research to identify more effective neuroprotective strategies. The combination of anti-inflammatory, antioxidant, and neurotrophic properties in such treatments might offer a more comprehensive approach to mitigating oxidative damage and promoting long-term recovery.

Effects of CPZ and other treatments on myelin basic protein (MBP)

MBP is a key structural component of myelin, and its levels serve as a critical indicator of myelination status. At week 5, the CPZ group exhibited significantly lower MBP levels (32.71 ± 2.26 pg/mg protein) compared to the CNT group (63.78 ± 7.84 pg/mg protein), confirming substantial demyelination induced by CPZ exposure [Figure 3]. The MBP levels in the BFB group were closer to those of the CNT group, suggesting that BFB treatment provided some degree of protection against myelin loss. In contrast, the CFB and CPB groups demonstrated significantly higher MBP levels than both the CPZ and CNT groups, highlighting the neuroprotective effects of these treatments in promoting myelination or preventing further demyelination.

After CPZ withdrawal, MBP levels in the CPZ group increased, suggesting spontaneous remyelination. However, despite this increase, MBP levels in the CPZ group did not fully return to baseline levels, indicating incomplete recovery of myelin. In the treatment groups, particularly CFB and CPB, MBP levels showed a slight decline at week 7, but they remained significantly higher than those in the CPZ group, which emphasizes the sustained protective effects of these treatments in promoting myelin preservation and regeneration.

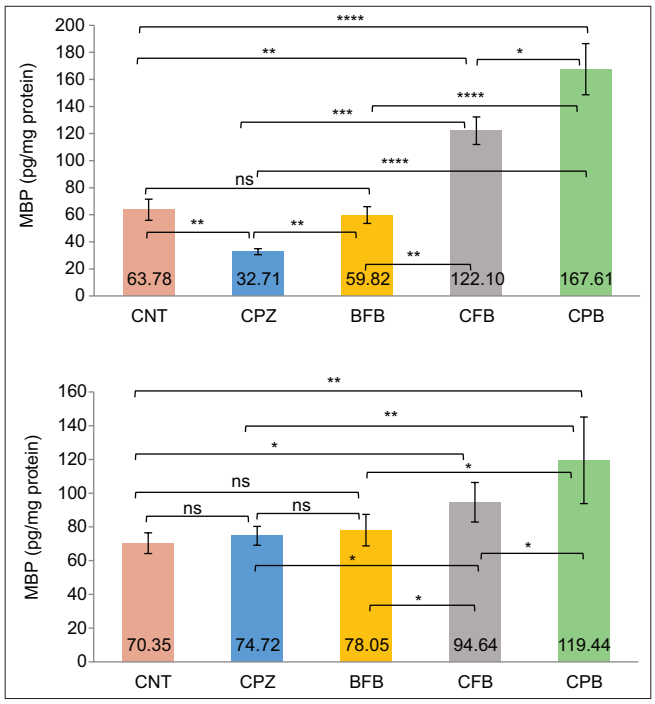

Figure 3: Level of myelin basic protein in the brainstem at the end of the demyelination stage (week 5) and at the end of the remyelination stage (week 7). One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$; NS: Non-significant

As MBP is an essential protein for myelin structure and function, its reduction reflects the loss of myelin integrity, which disrupts neuronal communication and can lead to long-term neurological impairments. This result is consistent with the well-established understanding that CPZ exposure leads to oligodendrocyte damage and demyelination, which is often accompanied by a significant decrease in MBP levels. The observation that the BFB group exhibited MBP levels closer to the CNT group suggests that BFB treatment may offer partial protection against myelin loss in brainstem [Figure 3]. This finding is supported by previous research showing that certain compounds can mitigate CPZ-induced demyelination by enhancing the survival of oligodendrocytes or promoting their differentiation. In contrast, the CFB and CPB groups exhibited significantly higher MBP levels than both the CPZ and CNT groups, indicating that these treatments not only prevented further demyelination but may have also enhanced myelination during the study period. The higher MBP levels in these groups further suggest that CFB and CPB may promote the differentiation or survival of oligodendrocyte precursor cells, leading to enhanced myelin regeneration.[21] The slight decline in MBP levels in the CFB and CPB groups at Week 7, although still significantly higher than in the CPZ group, suggests that these treatments may exert their effects early in the recovery phase but become less effective as time progresses. Nonetheless, the sustained higher MBP levels in these groups compared to CPZ provide strong evidence of their neuroprotective properties. These results are in line with studies demonstrating that neuroprotective agents can enhance both the survival of oligodendrocytes and the process of remyelination, particularly in the early stages of recovery.

Effects of CPZ and other treatments on inflammatory markers

Neuroinflammation is a hallmark of CPZ-induced toxicity, with key inflammatory markers, such as TNF-α, GFAP, IFN-γ, MIP-1, and CCL-5 being significantly elevated during demyelination. These markers provide valuable insights into the inflammatory and neuroprotective mechanisms of curcumin and piperine. TNF-α is a key pro-inflammatory cytokine involved in neuroinflammation and neurodegenerative processes. At the conclusion of the demyelination stage, TNF-α levels in the CPZ group (622.62 ± 51.26 pg/mg protein) were significantly elevated ($P \leq 0.01$) compared to the CTN group (398.44 ± 25.31 pg/mg protein), indicating severe neuroinflammation due to CPZ toxicity [Figure 4]. Similar findings have been reported in previous studies linking CPZ-induced toxicity to increased

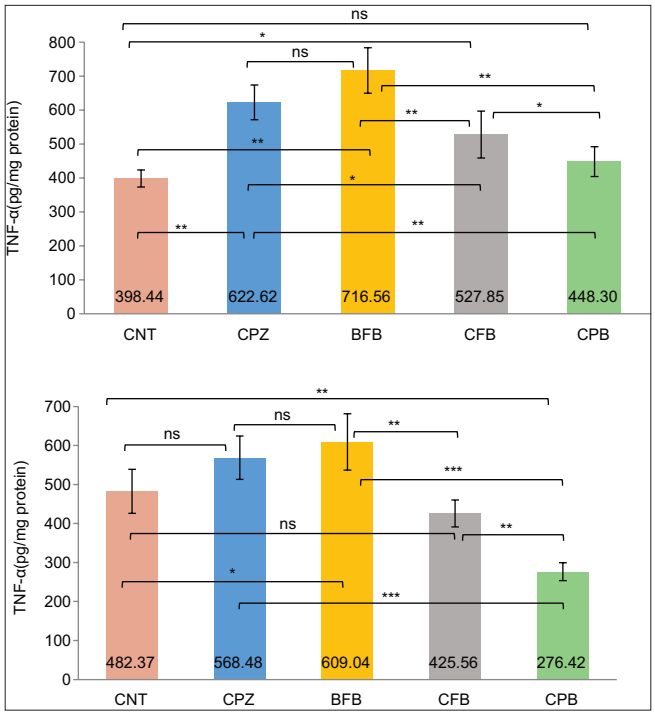

Figure 4: Level of tumor necrosis factor-alpha in the brainstem at the end of the demyelination stage (week 5) and at the end of the remyelination stage (week 7). One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$; NS: Non-significant

TNF-α expression.[30] The BFB group, which received blank formulation, exhibited a slightly higher but non-significant increase in TNF-α, suggesting that the BSO formulation did not provide significant protection against CPZ toxicity. Conversely, the CFB and CPB groups exhibited significantly lower TNF-α levels, suggesting that curcumin plays a crucial role in reducing inflammation. Notably, the CPB group demonstrated the lowest TNF-α level among the CPZ-exposed groups, indicating that piperine enhanced the anti-inflammatory effect of curcumin [Figure 4].

By the end of the study (week 7), TNF-α levels in the CPZ group had decreased (568.48 ± 69.87 pg/mg protein) from the week 5 level but remained elevated, indicating persistent inflammation [Figure 4]. A general decline in TNF-α was observed across all treatment groups. Although the BFB group exhibited a reduction compared to week 5, levels remained relatively high, suggesting slow recovery. The CFB and CPB groups demonstrated further reductions in TNF-α, with CPB showing the lowest levels (276.42 ± 23.18 pg/mg protein), reinforcing the neuroprotective synergy of curcumin and piperine. These findings align with previous research demonstrating the combined anti-inflammatory properties of curcumin and piperine.[31]

GFAP is a well-established marker of astrocyte activation and neuroinflammation. At week 5, the CPZ group exhibited the highest GFAP levels (94.74 ± 9.84 pg/mg protein), significantly elevated compared to the BFB, CFB, and CPB groups, consistent with astrocyte activation in response to CPZ-induced demyelination [Table 2]. The BFB group, which received the blank formulation, exhibited moderate GFAP levels (52.38 ± 6.92 pg/mg), significantly different from the CTN and CPB groups. Interestingly, the CPB group showed the lowest GFAP levels (28.71 ± 4.16 pg/mg), significantly lower than the CFB group, suggesting a strong anti-inflammatory effect of curcumin, further enhanced by piperine. By week 7, GFAP levels in the CPZ group remained elevated (75.34 ± 8.67 pg/mg) but showed partial recovery. The BFB group exhibited a slight increase (59.82 ± 6.g), but this was not significantly different from the CTN group, indicating some neuroprotection. The CFB and CPB groups showed further reductions in GFAP, reinforcing curcumin's anti-inflammatory role and the enhanced bioavailability effect of piperine.[32]

Five weeks of CPZ ingestion resulted in a significant increase in IFN-γ levels (247.10 ± 31.82 pg/mg) compared to the CTN and CFB groups. The BFB group exhibited moderate IFN-γ levels (173.07 ± 14.29 pg/mg), significantly different from the CTN and CPZ groups [Table 2]. The CPB group showed the lowest IFN-γ levels (75.29 ± 5.61 pg/mg), suggesting a potent anti-inflammatory effect. Upon cessation of CPZ at week 5, the CPZ group exhibited spontaneous recovery and remyelination, as indicated by a drop in IFN-γ levels (106.80 pg/mg). The BFB group also demonstrated a reduction, albeit higher than CFB and CPB. The CFB group exhibited a further reduction in IFN-γ (84.85 ± 6.94 pg/mg), significantly different from the CTN, CPZ, and BFB groups. The CPB group had the lowest IFN-γ level at week 7 (61.37 ± 5.22 pg/mg), significantly different from the CFB ($P \leq 0.05$) and CTN groups ($P \leq 0.05$), suggesting the strongest anti-inflammatory effect among all combinations, consistent with reports from prior research.[33]

MIP-1 is a chemokine associated with neuroinflammation. At the demyelination stage (week 5), the CPZ group had the highest MIP-1 levels (521.28 ± 44.36 pg/mg), significantly higher than all other groups, confirming a strong inflammatory response to CPZ exposure [Table 2]. The curcumin nanoformulations, CFB (160.46 ± 25.31 pg/mg) and CPB (136.71 ± 21.83 pg/mg), effectively mitigated the effects of CPZ toxicity, as evidenced by significantly lower MIP-1 levels compared to the CPZ group. CPZ withdrawal led to a significant reduction in MIP-1 levels in the CPZ group (392.61 ± 41.82 pg/mg), though levels remained higher than BFB, CFB, and CPB groups. By week 7, MIP-1 levels remained significantly reduced in CFB and CPB groups, indicating sustained anti-inflammatory effects of curcumin formulations, consistent with findings in experimental neuroinflammation models.

CCL-5, also known as RANTES, plays a key role in immune cell recruitment and neuroinflammation. CPZ administration for 5 weeks significantly increased CCL-5 levels compared to CTN, BFB, CFB, and CPB groups [Table 2], confirming

Table 2: Levels of GFAP, IFN-γ, MIP-1 and C-C motif CCL-5, in brainstem at the end of week 5 and week 7

Group	Week 5	*P*-value	Week 7	*P*-value
		GFAP (pg/mg protein)		
CNT	39.33±4.68	***CNT×CPZ, CPB	54.21±5.48	**CNT×CPZ, CFB
CPZ	94.74±9.84	**CPZ×BFB, CFB ***CPZ×CPB	75.34±8.67	***CPZ×CPB **CPZ×CFB
BFB	52.38±6.92	*BFB×CNT, CPB	59.82±6.84	NS=BFB×CNT
CFB	45.62±5.27	NS=CFB×BFB	38.24±4.21	*CFB×BFB
CPB	28.71±4.16	*CPB×CFB	25.77±5.93	**CPB×CNT
		IFN-γ (pg/mg protein)		
CNT	119.88±12.14	*CNT×CPB	113.45±8.41	NS=CNT×CPZ, BFB
CPZ	247.10±31.82	***CPZ×CNT, CFB	106.80±7.78	**CPZ×CPB
BFB	173.07±14.29	*BFB×CNT, CPZ	111.75±13.46	**BFB×CPB
CFB	91.54±7.52	**CFB×BFB; *CFB×CNT	84.85±6.94	*CFB×CNT, CPZ, BFB
CPB	75.29±5.61	NS=CFB×CPB	61.37±5.22	*CPB×CFB; **CPB×CNT
		MIP-1 (pg/mg protein)		
CNT	232.25±26.78	***CNT×CPZ	238.96±39.47	**CNT×CPZ
CPZ	521.28±44.36	****CPZ×CFB, CPB	392.61±41.82	***CPZ×BFB, CFB, CPB
BFB	272.77±26.49	**BFB×CPZ, CPB, CFB	144.40±11.26	NS=BFB×CFB, CPB
CFB	160.46±25.31	NS=CFB×CPB	168.69±24.49	*CNT×CPB, CFB, BFB
CPB	136.71±21.83	*CPB×CNT	153.41±25.48	NS=CPB×CFB
		CCL-5 (pg/mg protein)		
CNT	783.64±65.45	**CNT×CPZ	906.57±81.96	**CNT×CPB; NS=CNT×CFB
CPZ	1196.86±112.85	***CPZ×CPB; *CPZ×CFB	1365.19±142.15	*CPZ×CNT, CFB
BFB	756.35±87.52	**BFB×CPZ	764.42±86.41	***BFB×CPZ; **BFB×CPB
CFB	821.75±113.25	NS=CFB×BFB; *CFB×CPB	964.88±94.38	*CFB×CPB
CPB	569.18±96.48	*CPB×CNT, BFB	499.73±68.76	****CPB×CPZ

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. One-way analysis of variance with Tukey's multiple comparison test was done to compare the variance between groups. NS: Non-significant, GFAP: Glial Fibrillary Acidic Protein, TNF-α: Tumor necrosis factor-alpha, IL-1β: Interleukin-1β, IFN-γ: Interferon-gamma, MIP-1: Macrophage inflammatory protein-1, CCL-5: C-C motif chemokine ligand 5, CNT: Control, CPZ: Cuprizone, BFB: Blank/vehicle formulation of black seed oil, CFB: Nanoformulation of curcumin in black seed oil, CPB: Curcumin-piperine formulation in black seed oil

neuroinflammation. The CPB group exhibited the lowest CCL-5 levels (569.18 ± 96.48 pg/mg), significantly lower than other groups except for CFB, highlighting the protective role of curcumin-piperine combinations. By week 7, CCL-5 levels in the CPZ group further increased (1365.19 ± 142.15 pg/mg), significantly higher than CTN and CFB groups, indicating persistent inflammation despite CPZ withdrawal. The BFB group showed a modest increase (764.42 ± 86.41 pg/mg), remaining lower than CPZ but higher than CPB. The CFB group also exhibited an increase (964.88 ± 94.38 pg/mg) and was significantly higher than CPB. Notably, CPB further reduced CCL-5 levels (499.73 ± 68.76 pg/mg), significantly different from CPZ ($P \leq 0.0001$), reinforcing its strong anti-inflammatory potential.[34]

CONCLUSION

This study demonstrated the neurotoxic effects of CPZ-induced demyelination in the brainstem, characterized by oxidative stress, neuroinflammation, and reduced myelin integrity. CPZ exposure significantly impaired antioxidant enzyme activity (catalase and SOD), elevated lipid peroxidation (MDA), increased pro-inflammatory cytokines (TNF-α, IFN-γ, GFAP, MIP-1, and CCL-5), and reduced MBP levels, confirming severe neurodegeneration. Treatment with curcumin and piperine nanoformulations in BSO showed promising neuroprotective effects. The curcumin-piperine combination exhibited superior efficacy in reducing oxidative stress, modulating inflammatory responses, and promoting remyelination compared to curcumin alone. The nanoformulation significantly restored antioxidant enzyme activity reduced inflammatory markers, and improved MBP levels, suggesting enhanced neuroprotection and myelin repair. These findings highlight the therapeutic potential of curcumin-piperine nanoformulations in mitigating MS-related neurodegeneration. Further research is needed to optimize bioavailability and evaluate long-term efficacy in preclinical and clinical settings. The combination

of anti-inflammatory, antioxidant, and neuroprotective properties suggests that curcumin-piperine nanoformulations could serve as a promising adjunct therapy for MS and other neurodegenerative disorders.

DATA AVAILABILITY STATEMENT

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

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