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Berberine protects against behavioral deficits, memory dysfunction, and callosal demyelination in cuprizone-intoxicated C57BL/6 mice

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ABSTRACT

Objective(s): Multiple sclerosis (MS) is a neurological disorder marked by progressive demyelination and neuroinflammation. Berberine, a plant-derived isoquinoline alkaloid found in Berberis species, has notable anti-inflammatory and antioxidant properties. This study aimed to evaluate berberine's protective effects against cuprizone intoxication in C57BL/6 mice.

Materials and Methods: C57BL/6 mice received a cuprizone-containing diet (0.2% w/w) for five weeks and were concurrently treated with berberine (5 or 10 mg/kg). Body weight, motor coordination, exploratory and locomotor activity, memory function, oxidative stress biomarkers, and inflammatory cytokines were assessed. Histopathological evaluation of myelination was performed using Luxol Fast Blue staining.

Results: Berberine treatment improved exploratory behavior, locomotor activity, and memory function compared with cuprizone-only mice. Oxidative stress analysis showed that berberine significantly reduced malondialdehyde (MDA) levels and increased total thiol content. Berberine also attenuated cuprizone-induced increases in interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Histological findings indicated that berberine preserved myelin integrity, prevented myelin degradation, and reduced the severity of demyelination in the corpus callosum.

Conclusion: These findings indicate that berberine confers protective effects against cuprizone-induced behavioral deficits, memory dysfunction, and callosal demyelination in C57BL/6 mice. The neuroprotective actions of berberine appear to be mediated by suppression of oxidative stress and inflammatory cytokines.

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Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated degenerative disorder characterized by inflammation within the central nervous system (1, 2). Pathological hallmarks of MS include the loss of oligodendrocytes and myelin-forming cells, activation of microglia and astrocytes, and infiltration of peripheral immune cells (3, 4).

Demyelination ultimately leads to axonal and neuronal degeneration, which underlies many of the neurological deficits associated with MS (5). Although the precise pathogenesis of MS remains unclear, oxidative stress, mitochondrial dysfunction, and neuroinflammation, alongside immune cell dysregulation, are widely recognized as key contributors to both axonal injury and myelin loss (6, 7).

Cuprizone (bis(cyclohexanon)oxaldihydrazon) is a neurotoxic, selective, and potent copper chelator (8). The cuprizone model is particularly valuable for studying mechanisms of demyelination and spontaneous remyelination (9).

Copper serves as a cofactor in numerous enzymatic and metabolic processes, including mitochondrial electron transport and antioxidant defense pathways (10). Cuprizone exposure disrupts cytochrome function and reduces monoamine oxidase (MAO) activity, thereby promoting mitochondrial oxidative stress and initiating

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localized immune responses. Mature oligodendrocytes are the primary targets of cuprizone toxicity, resulting in oligodendroglial cell death and extensive demyelination throughout the telencephalon. The posterior corpus callosum, hippocampus, and superior cerebellar peduncles are among the most severely affected regions (11). A cuprizone-containing diet over 5-6 weeks induces behavioral abnormalities, impaired motor function, and altered mood in rodents, while withdrawal of cuprizone typically triggers spontaneous remyelination within approximately 12 weeks (12).

Berberine is a bioactive isoquinoline alkaloid derived from *Berberis* species, long used in traditional medicine and as a natural food additive (13). It exhibits diverse pharmacological properties, including anti-inflammatory, antipyretic, antidepressant, hypoglycemic, and lipid-lowering effects, and is generally considered safe at therapeutic doses, with no cytotoxic, genotoxic, or mutagenic activity (14).

Berberine demonstrates neuroprotective effects in multiple experimental models of neurological disease, including Alzheimer's disease (15, 16), middle cerebral artery occlusion (MCAO) (17), and cerebral ischemia in gerbils (18). It promotes neuronal survival and neurite outgrowth in SH-SY5Y cells and hippocampal precursor cells (19, 20) and enhances axonal regeneration in models of sciatic nerve injury. Moreover, berberine has beneficial immunomodulatory effects in autoimmune disorders such as type 1 diabetes (21), Rheumatoid arthritis (22), and experimental autoimmune encephalomyelitis (EAE) (23-25).

The therapeutic potential of berberine is attributed to its antioxidant properties, suppression of reactive oxygen species, reduction of intracellular calcium, inhibition of N-methyl-D-aspartate (NMDA) receptor overactivation, and potent anti-inflammatory actions (15, 26, 27). Berberine modulates inflammation through multiple intracellular signaling pathways.

Given these properties, we hypothesized that berberine could attenuate cuprizone-induced demyelination in C57BL/6 mice. To test this hypothesis, we evaluated its effects on balance and motor performance, spatial memory, histopathological changes, oxidative stress markers, and inflammatory cytokines.

Materials and Methods

Animals

Male C57BL/6 mice, 7 weeks old and weighing approximately 20 g, were used in this study. Animals were housed in groups of seven per cage under standard laboratory conditions, including a 12 hr light/dark cycle, controlled temperature (23 ± 2 °C), and ad libitum access to food and water. All experimental procedures were approved by the Animal Experimental Ethics Committee for the Care and Use of Laboratory Animals at Mashhad University of Medical Sciences (Approval No. IR.MUMS.MEDICAL.REC.1397.076) and complied with the National Institutes of Health guidelines for laboratory animal care and use.

Induction of demyelination

Following a period of acclimatization, mice were randomly assigned to four groups:

Control: received standard diet for 35 days.

Cuprizone: received a diet containing 0.2% (w/w) cuprizone mixed with normal chow for 35 days. They received 1% DMSO in normal saline intraperitoneally as the injection vehicle for 35 days.

Cuprizone + Berberine 5 mg/kg: received intraperitoneal (i.p.) berberine (5 mg/kg/day) concurrently with cuprizone diet for 35 days.

Cuprizone + Berberine 10 mg/kg: received berberine (10 mg/kg/day, IP) concurrently with cuprizone diet for 35 days.

At the end of the treatment period, mice underwent behavioral assessments (rotarod and open field tests) and were subsequently subjected to histological and biochemical analyses (28).

Body weight measurement

Body weight was recorded daily at 10:00 throughout the 35-day experimental period.

Behavioral Tests

After completion of the cuprizone exposure period, behavioral functions were assessed. All equipment was cleaned with 70% ethanol between tests.

Rotarod test

Motor coordination and balance were evaluated using a rotarod apparatus. Mice were trained for 5 min daily for three consecutive days. On day 35, mice were placed on the rotating rod (initial speed of 10 rpm) and accelerated to 20 rpm over 20 sec. The latency to fall was recorded, with a maximum cut-off time of 300 sec (29).

Open field test

Locomotor activity was examined on day 35 in an open-field arena (45 × 45 × 45 cm) composed of a Plexiglas box with black walls and a white floor divided into 25 squares (9 × 9 cm). Each mouse was placed in the central square and allowed to explore freely for 300 sec. Movements were recorded and analyzed using EthoVision XT11 video-tracking software (Noldus, Netherlands) (30).

Y-maze test

Spatial working memory was assessed using a Y-maze consisting of three arms (21 × 7 × 15.5 cm) positioned at 120° angles (31, 32). Each mouse was placed at the center and allowed to explore for 8 min. Spontaneous alternation was defined as consecutive entry into three different arms (e.g., ABC, BCA, CAB). The percentage of alternation was calculated using: Spontaneous Alternation (%) = [Number of Alternations / Total Arm Entries-2] × 100.

The maze was cleaned with 70% ethanol between trials.

Tissue collection

On day 35, mice were euthanized via intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg). The brains were rapidly removed and fixed in 4% paraformaldehyde (PFA) at 4 °C for 72 hr. Following fixation, the brains were embedded in paraffin wax. Coronal sections (5 µm) were then cut using a microtome and mounted on glass slides.

Additional samples of the corpus callosum were micro-dissected, homogenized in PBS (pH 7.0), and stored at -20 °C for biochemical analysis.

Luxol fast blue (LFB) staining

Demyelination in the corpus callosum was assessed using LFB staining. Paraffin-embedded sections were deparaffinized and stained with 1% LFB (in 95% ethanol

with 0.05% acetic acid) at 60 °C for 2 hr. Sections were differentiated in 0.05% lithium carbonate for 20 sec, then washed in 70% ethanol, and counterstained with 0.1% Cresyl Fast Violet for 4 min. After dehydration and clearing in xylene, slides were mounted for microscopic evaluation (33).

Measurement of oxidative stress parameters

Malondialdehyde (MDA)

Lipid peroxidation was quantified using the thiobarbituric acid reactive substances (TBARS) assay. Briefly, 1 ml of brain homogenate was mixed with 2 mL of TBA/TCA/HCl reagent (0.37% TBA, 15% TCA, 0.2 N HCl) and incubated in a boiling water bath for 40 min. After cooling and centrifugation (1000 rpm, 10 min), supernatant absorbance was measured at 532 nm (34).

Thiol content

Thiol content in the tissue was measured. A 0.5 mL aliquot of the homogenate was mixed with 1 mL of Tris-EDTA buffer (pH 8.6). The supernatant was combined with 2 mL of phosphate buffer (0.1 M, pH 7.4) and 0.5 mL of 0.3 mM 5,5′-Dithiobis-(2-nitrobenzoic) acid (DTNB). Absorbance was recorded at 412 nm within 10 min (35).

Detection of inflammatory cytokines

Levels of IL-1β and TNF-α in homogenates were quantified using sandwich ELISA kits according to the manufacturer's instructions. Results were expressed as pg/mg protein.

Statistical analysis

All data were given as means ± standard deviation (SD). The obtained data was analyzed using one-way analysis of variance (ANOVA) with Tukey-Kramer *post hoc* test by GraphPad Prism 6.01 (La Jolla, CA, USA) software. In all tests, $P<0.05$ was considered significant.

Results

Berberine effects on weight alteration in cuprizone-treated mice

At baseline, no significant differences in body weight were observed among the experimental groups. By the end of the study, cuprizone-fed animals exhibited a slight but significant reduction in body weight ($P<0.001$) compared with the control group (Figure 1A-B). Weight loss was also observed in the berberine-treated groups; however, it was less pronounced in the cuprizone + berberine 5 mg/kg ($P<0.01$) and cuprizone + berberine 10 mg/kg ($P<0.05$) groups than in cuprizone alone.

Berberine impacts on motor coordination and balance ability in cuprizone-treated mice

As shown in Figure 2, cuprizone-fed animals exhibited a significant reduction in latency to fall on the rotarod test compared with controls ($P<0.001$). Treatment with berberine at 10 mg/kg significantly ameliorated this deficit ($P<0.05$), indicating improved motor coordination and balance.

Berberine improves anxiety and exploratory ability in cuprizone-treated mice

Cuprizone administration significantly decreased locomotor speed ($P<0.001$) and reduced the time spent in the central arena of the open-field apparatus ($P<0.001$),

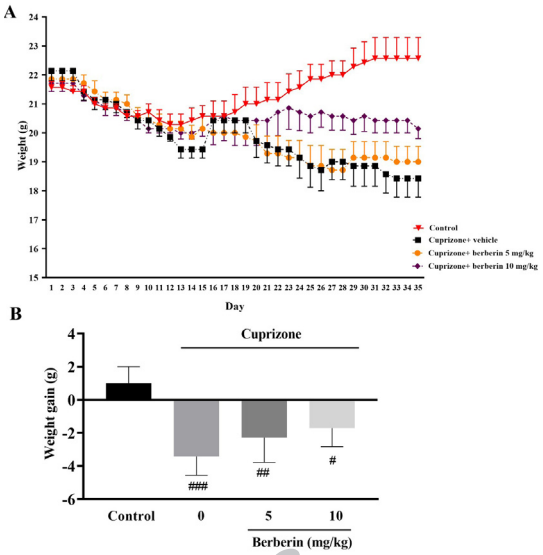

Figure 1. Effects of berberine on
(A) body weight changes, and (B) weight gain following 35 days of cuprizone administration in C57BL/6 mice. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs. control group

indicating anxiety-like and hypoactive behavior (Figure 3A-B). Berberine at 5 mg/kg significantly increased the time spent in the center, whereas the 10 mg/kg dose significantly improved both locomotor speed ($P<0.001$) and central zone duration ($P<0.001$). Representative motion-tracking patterns are shown in Figure 3C.

Berberine ameliorates working memory impairment in cuprizone-treated mice

As shown in Figure 4, cuprizone exposure markedly reduced spontaneous alternation in the Y-maze compared with the control group (47.52 ± 5.08% vs. 75.82 ± 6.90%). Berberine at 10 mg/kg significantly restored alternation performance (59.53 ± 7.60%, $P<0.05$), whereas berberine at 5 mg/kg showed a non-significant trend toward improvement.

Berberine alters biochemical parameters in cuprizone-treated mice

Cuprizone administration significantly increased MDA levels (30.30 ± 3.60% vs. 21.65 ± 2.05% in controls, $P<0.01$; Figure 5A). Treatment with berberine at 5 and 10 mg/kg significantly reduced MDA concentrations ($P<0.05$ and $P<0.01$, respectively), bringing them closer to control values (24.18 ± 3.31% and 21.45 ± 1.68%, respectively).

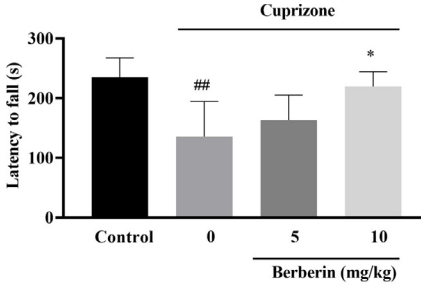

Figure 2. Effect of berberine on latency to fall in the rotarod test following 35 days of cuprizone administration in mice
Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. ##$P<0.01$ compared to the control group; *$P<0.05$ compared to the cuprizone group.

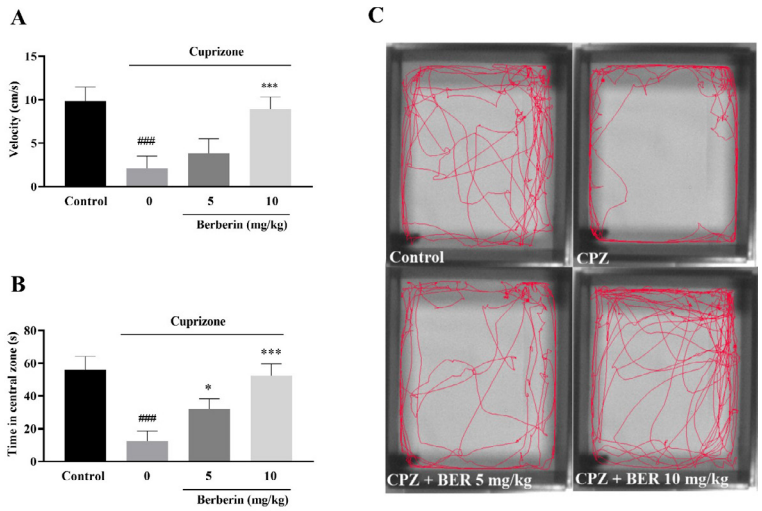

Figure 3. Effects berberine on:
(A) average speed (velocity), (B) time spent in the central zone, and (C) locomotor tracking recorded by a video-tracking camera syste[illegible] in the open field test following 35 days of cuprizone administration. Data are presented as mean±SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.
###*P*<0.001 compared to the control group; **P*<0.05 and ****P*<0.001 compared to the CPZ group.
CPZ: Cuprizone; BER: Berberine

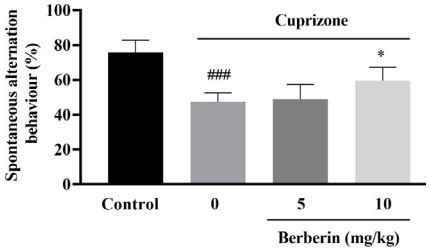

Figure 4. Effect of berberine on spontaneous alternation behavior in th[illegible] Y-maze test following 35 days of cuprizone administration in mice
Data are presented as mean±SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.
###*P*<0.001 compared to the control group; **P*<0.05 compared to the cupri[illegible] g[illegible]

In addition, cuprizon[illegible] significantly decreased thiol level compared with the control group (12.80 ± 2.15 vs. 18.40 ± 0.74, *P*<0.01; Figure 5B). Berberine at 10 mg/kg significantly increased thiol levels (16.58 ± 1.30%, *P*<0.05), whereas the 5 mg/kg dose produced a non-significant elevation.

Effect of berberine on inflammatory cytokines in cuprizone-treated mice

Cuprizone intoxication markedly elevated IL-1β and TNF-α levels by approximately 3.26-fold (112.70 ± 13.61 vs. 34.52 ± [illegible] pg/mg protein, *P*<0.001) and 4.59-fold (97.93 ± 11.07 vs. 21.37 ± 5.63 pg/mg protein, *P*<0.001), respectively (Figure 6). Berberine at 5 mg/kg significantly reduced IL-1β (*P*<0.05) and TNF-α (*P*<0.05). The 10 mg/kg dose produced even greater

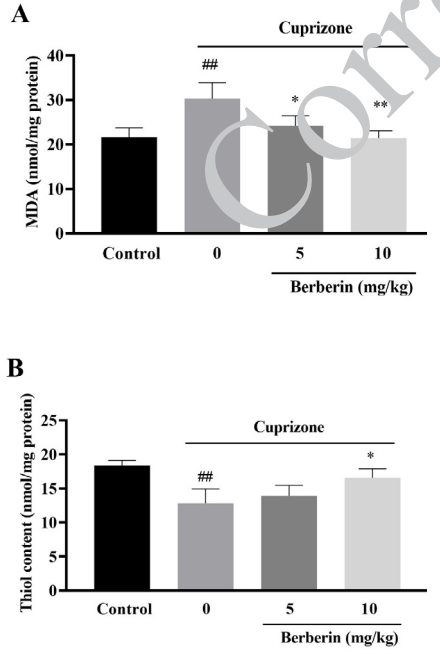

Figure 5. Effects of berberine on:
(A) malondialdehyde (MDA) and (B) sulfidryl mouse groups following 35 days of cuprizone administration. Data are presented as mean±SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.
##*P*<0.01 compared to the control group; **P*<0.05 and ***P*<0.01 compared to the cuprizone group.

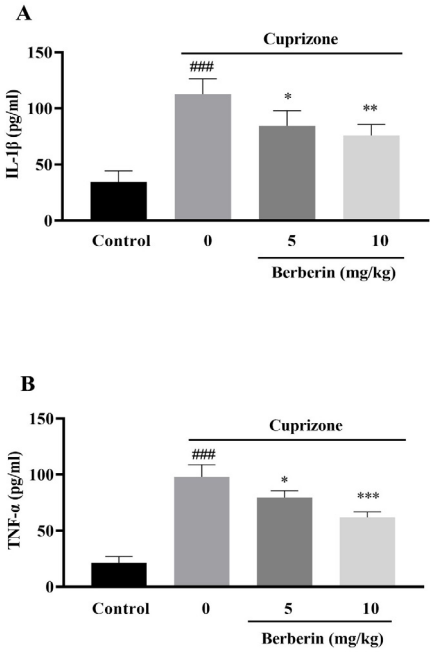

Figure 6. Effects of berberine on (A) IL-1β and (B) TNF-α levels following 35 days of cuprizone administration in mice
Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. ###*P*<0.001 compared to the control group; **P*<0.05, ***P*<0.01, and ****P*<0.001 compared to the cuprizone group.

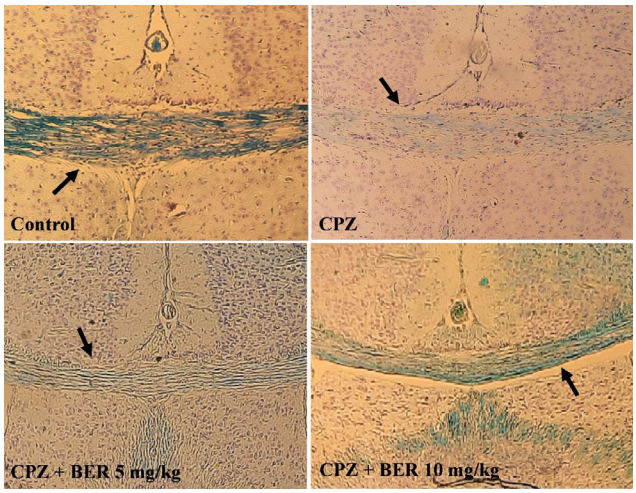

Figure 7. Microscopic examination of the CC in mouse experimental groups stained with LFB under light microscopy (10× magnification)
The arrows show demyelination on LFB stained sections in the CC. Pronounced decrease in the positive fiber staining was shown in the CC from cuprizone CPZ-treated mice, compared to naïve brains. In the brains from CPZ-fed mice that were treated with berberine BER, the loss of fibers staining was markedly ameliorated. CPZ: Cuprizone; BER: Berberine; LFB: Luxol fast blue; CC: Corpus callosum

reductions in IL-1β (P<0.01) and TNF-α (P<0.001).

Berberine improves demyelinating lesions in cuprizone-treated mice

LFB staining revealed extensive demyelination in the corpus callosum of cuprizone-treated animals, as indicated by diminished blue myelin staining (Figure 7). Berberine administration substantially mitigated demyelinating lesions, demonstrating preservation of myelin integrity and reduced cuprizone-induced neuropathology.

Discussion

This study investigated the protective effects of berberine in a cuprizone-induced demyelination model in C57BL/6 mice. Our findings demonstrated that berberine effectively mitigated behavioral impairments and restored biochemical and histological parameters altered by cuprizone exposure.

Consistent with previous reports, cuprizone-fed animals exhibited reduced body weight (36, 37). This weight loss has been attributed to reduced food intake due to cuprizone's aversive taste, as well as disruptions in normal metabolic function and energy homeostasis (38). Although berberine treatment attenuated several cuprizone-induced deficits, it did not significantly reverse body weight loss. This may be associated with berberine's known appetite-suppressant effects mediated through central pathways regulating obesity (39, 40).

In the open-field box, mice that received cuprizone spent more time in the peripheral area. The animals' speed also decreased. These results were confirmed by Zahednasab et al. (41). This phenomenon could be the consequence of cuprizone's influence on white matter, producing anxiety-like behavior (42). Furthermore, impairments in motor coordination and balance were observed in cuprizone-treated animals, consistent with previous evidence linking demyelination to sensorimotor dysfunction (43, 44).

Cuprizone markedly reduced spontaneous alternation behavior in the Y-maze test, indicating deficits in spatial working memory. This impairment reflects dysfunction in the hippocampus and prefrontal cortex, regions vulnerable to cuprizone-induced oligodendrocyte loss, myelin disruption, and neuroinflammation. Prior research supports these observations and further validates the cuprizone model as an appropriate tool for studying demyelination-associated cognitive impairment (45, 46).

Berberine demonstrated a significant capacity to counteract these alterations. Numerous preclinical studies have shown that berberine improves cognitive performance and attenuates neurobehavioral disturbances in models of Alzheimer's disease, schizophrenia, and other neurodegenerative conditions (47-49).

Histological findings in the present study revealed profound demyelination of the corpus callosum after cuprizone administration. Cuprizone induces region-specific oligodendrocyte death and subsequent demyelination while causing minimal blood-brain barrier disruption or peripheral immune infiltration. This pattern is well documented in prior studies of the corpus callosum, basal ganglia, and cerebellar nuclei (50-52). Oligodendrocytes are particularly sensitive to metabolic stress because of their high demand for lipid and protein synthesis and limited antioxidant capacity (53, 54). Consequently, mitochondrial dysfunction and excessive ROS production are considered key mechanisms underlying cuprizone toxicity (55).

Our biochemical analyses confirmed oxidative stress in cuprizone-treated mice, showing reduced thiol content and elevated MDA levels, consistent with previous studies (36, 56, 57). Oxidative injury is a central factor in oligodendrocyte vulnerability, lipid peroxidation, and neuronal membrane damage.

Berberine appears to counteract this pathological cascade through multiple complementary mechanisms. First, its antioxidant effect may involve direct free radical scavenging; more importantly, it likely reflects activation of endogenous cytoprotective pathways, particularly nuclear factor erythroid 2–related factor 2 (Nrf2) signaling. Berberine has been identified as a pharmacological activator of the Nrf2 pathway, a critical endogenous defense mechanism against cuprizone-induced oxidative injury. By enhancing antioxidant responses and preserving glutathione homeostasis, berberine may effectively attenuate lipid peroxidation and reduce oligodendrocyte vulnerability (47, 58-60). These antioxidant mechanisms align with the observed improvements in MDA and thiol levels in berberine-treated, cuprizone-intoxicated mice.

In addition to oxidative damage, cuprizone markedly increased IL-1β and TNF-α levels, consistent with previous observations in demyelination models (61, 62). These cytokines are prominent mediators of neuroinflammation and play central roles in the pathogenesis of MS. The observed reduction in the pro-inflammatory cytokines IL-1β and TNF-α following berberine treatment suggests modulation of the neuroinflammatory milieu. Comparable anti-inflammatory effects have been demonstrated in cuprizone-fed rats treated with berberine-loaded nanoparticles (63) and in murine EAE models, where berberine attenuated clinical severity, protected the blood-brain barrier, suppressed pro-inflammatory cytokines, and enhanced anti-inflammatory responses (23, 24, 64).

Mechanistically, these anti-inflammatory effects may be mediated by suppressing canonical inflammatory signaling pathways, including nuclear factor kappa B (NF-κB), a major regulator of IL-1β and TNF-α transcription. In microglia, inhibition of NF-κB signaling may shift polarization away from the pro-inflammatory M1-like

phenotype toward an anti-inflammatory and reparative M2-like phenotype. This phenotypic transition is particularly relevant in demyelinating disorders, as activated microglia can either exacerbate tissue injury or facilitate myelin debris clearance and support remyelination, depending on their functional state. Recent evidence further suggests that berberine promotes microglial polarization toward an M2-like phenotype and enhances phagocytic activity through mechanisms involving TYROBP activation (49). Collectively, these findings support the concept that berberine modulates microglial function and attenuates immune-mediated damage in demyelinating conditions. This multimodal mechanism may ultimately contribute to preservation of myelin integrity and improvement of neurobehavioral outcomes.

Conclusion

Taken together, the present findings demonstrate that berberine exerts significant protective effects against cuprizone-induced demyelination in mice. Berberine alleviated behavioral deficits, improved markers of oxidative stress, reduced pro-inflammatory cytokine levels, and attenuated histological damage in the corpus callosum. These results suggest that berberine has therapeutic potential for demyelinating disorders such as MS, particularly because of its combined antioxidant, anti-inflammatory, and neuroprotective properties.

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Authors' Contributions

HR S provided concept and design. MS A, SF H, M A, N LK and E M performed data collection and processing and conducted the experiment. MS A and N LK analyzed and interpreted the results. MS A, SF H, and F F prepared the manuscript draft. A AG and HR S critically revised the article. HRS obtained final approval, provided supervision, and secured funding.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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