Cytokine 113 (2019) 417–426

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

The protective effect of rifampicin on behavioral deficits, biochemical, and neuropathological changes in a cuprizone model of demyelination

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ARTICLE INFO

Keywords:
Multiple sclerosis
Demyelination
Rifampicin
Cuprizone
Corpus callosum
Cell death

ABSTRACT

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) in which both neuroinflammation and neurodegeneration play critical roles in the pathogenesis of the disease. A growing body of evidence indicates that some antibiotics have anti-inflammatory and neuroprotective properties. Rifampicin, commonly used for the treatment of mycobacteria, has been shown to exert neuroprotective activities in neurodegenerative diseases. In this study, we examined the efficacy of rifampicin on demyelination, gliosis, apoptosis, inflammation, behavioral dysfunction, and biochemical alterations in the cuprizone model of demyelination. For this aim, male C57BL/6J mice were fed a chow containing 0.2% cuprizone (w/w) for 6 weeks to induce reversible demyelination in the corpus callosum. Mice intraperitoneally received serial doses of rifampicin (10, 20, or 40 mg/kg body weight) in the last 7 days of a 6-week period of cuprizone treatment. The results showed that the administration of rifampicin led to the improvement in motor behavioral deficits. In line with this, rifampicin decreased the number of apoptotic cells in the corpus callosum thereby diminishing the expression of cleaved caspase-3 and Bax, as well as increasing Bcl-2. Moreover, rifampicin significantly lowered the levels of interleukin-6, interleukin-1β, caspase-12 activity, heme oxygenase-1(HO-1), nitric oxide (NO), and malondialdehyde (MDA) in mice treated with cuprizone. Conversely, the activity of glutathione peroxidase (GPx) and the level of ferric reducing ability of plasma (FRAP) were increased in response to the treatment with rifampicin. Histopathological findings demonstrated that rifampicin statistically promoted remyelination and mitigated microgliosis and astrogliosis. It seems that rifampicin is able to be added to the armamentarium of therapies for multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) in which both the brain and spinal cord are attacked by the immune system [1]. The hallmark of the pathological features of MS is the presence of demyelinated lesions caused by immune insults and oligodendrocyte loss [2]. The infiltrating immune cells including T and B cells along with the resident microglia in the CNS secret the proinflammatory cytokines including interleukine-1β (IL-1β) and interleukin-6 (IL-6) which are responsible for cell death and axonal damage. The symptoms of MS vary, depending on where the lesions occur in the CNS [3]. Additionally, the formation of reactive oxygen species (ROS) is involved in the progression of neural damage and destruction of the myelin sheath in MS disease [4]. There are some rodent models for studying MS pathology that help researchers to understand the pathological characteristics of the disease. Experimental autoimmune encephalomyelitis (EAE) and cuprizone-induced demyelination are two prominent models used to study the detrimental role of the immune system and the demyelination/remyelination process in MS, respectively [5]. Intoxication with cuprizone in mice for a 6-week period results in the reversible destruction of the myelin sheath mainly occurring in the corpus callosum followed by the activation of microglia and astrocytes [6].

Rifampicin is a semi-synthetic antibiotic that is widely prescribed

Abbreviations: MS, multiple sclerosis; ECL, enhanced chemiluminescence; MDA, malondialdehyde; PVDF, Polyvinylidene difluoride; PAFA, paraformaldehyde; DMSO, dimethyl sulfoxide; TUNEL, transferase-mediated dUTP nick-end labeling; NO, nitric oxide; BCA, bicinchoninic acid; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power; EAE, experimental autoimmune encephalomyelitis; GPx, glutathione peroxidase

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https://doi.org/10.1016/j.cyto.2018.10.016
Received 22 June 2018; Received in revised form 16 September 2018; Accepted 18 October 2018
Available online 24 October 2018
1043-4666/

for the treatment of leprosy and tuberculosis [7]. In addition to its intrinsic antibacterial properties, a myriad number of studies have shown that rifampicin acts as a neuroprotective and anti-inflammatory agent against various neuroinflammatory and neurodegenerative diseases [8]. It has been reported that rifampicin has beneficial effects on the attenuation of rotenone-induced microglial activation through oppressing NLRP3 inflammasome [9]. The advantages of rifampicin are not exclusively confined to dampening inflammation; rather, its neuroprotective effects have enabled this drug to halt α-synuclein fibrillation and disaggregate fibrils as well [10]. In line with this, the administration of rifampicin in patients with Alzheimer's disease enhances dysfunctional behavior, depression, and functional status [11].

In the present study, we tested the possible efficacy of rifampicin in the amelioration of the demyelination process occurring as a result of cuprizone intoxication in mice.

2. Materials and methods

2.1. Animals and demyelination induction

Sixty-four male C57BL/6J mice were procured from the Pasteur Institute, Tehran, Iran, weighing between 18 and 22 gr (7–9 weeks old). All animals were housed in the temperature (22 °C) and humidity (45%) controlled rooms with 12-h light cycles, and they had access to food and water *ad libitum*. In order to induce demyelination, mice were nourished with a diet containing 0.2% cuprizone mixed into ground standard rodent chow for 6 weeks. Control mice were kept on the same diet without cuprizone. In order to analyze factors associated with the corpus callosum, mice were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg); then, they were transcardially perfused with 0.9% saline and 10% formalin solution; then, they were decapitated, and the brains were removed. The study procedures were performed according to the Ethical Committee of Iran University of Medical Sciences. The ethical code assigned for this study was # IR.IUMS.REC 1395.27347.

2.2. Study design

Mice were randomly divided into four main groups: (i) control group which received normal powdered chow for 6 weeks with intraperitoneal (i.p.) injection of 10% dimethyl sulfoxide (DMSO) solution (diluted in phosphate-buffered saline) for the last 7 days of demyelination induction (n = 8); (ii) cuprizone group nourished with powdered chow that was mixed with 0.2% cuprizone for 6 weeks and received i.p. injection of 10% DMSO solution for the last 7 days of intoxication period (n = 8); (iii) rifampicin group (on 0.2% cuprizone diet) that was divided into three separate sub-groups, treated with 10, 20, or 40 mg/kg body weight of rifampicin (i.p.) during the last 7 days of the 6-week cuprizone feeding period (eight mice per sub-group); and (iv) healthy group which was divided into three sub-groups and received three doses of rifampicin (10, 20, or 40 mg/kg body weight) separately for 7 days (eight mice per subgroup). Rifampicin was administered once a day at 10:00 A.M. All mice from different groups were investigated by behavioral, molecular, biochemical, and histopathological examinations.

2.3. Chemicals and drugs

Rifampicin (CAS No. 13292-46-1) and cuprizone (CAS No.370-81-0) were purchased from Sigma (Sigma, St. Louis, MO, USA). The ELISA kits specified for the detection of HO-1 (ab204524), caspase-12 (ab65664), IL-1β (ab197742), IL-6 (ab100712) along with antibodies specified for β-actin (ab49900), Bax (ab32503), GFAP (ab7260), Iba-1 (ab178846), and MBP (ab77895) were procured from Abcam (Abcam, Cambridge, MA). The TUNEL kit was procured from AATBio (AAT Bio., Sunnyvale, CA, USA). Mouse Bcl-2 ELISA kit was purchased from Antibodies-online (Antibodies-online, Inc, GA, USA). Anti-mouse cleaved caspase-3 (Cat No. MAB835) was purchased from R&D Systems (R&D Systems, Minneapolis, MN). ECL (Cat No. RPN2232) kit was obtained from Amersham Bioscience (Little Chalfont Bucks, UK). The secondary antibody (ab97046) used in this study was purchased from Abcam (Abcam, Cambridge, MA).

3. Behavioral experiments

3.1. Open field

At the end of the experiment period, the effect of rifampicin on exploratory activity was assessed using the open-field test. Mice (each group, n = 8) were placed in an open-field apparatus, and locomotion was monitored over a 5-min period. The apparatus was made of a 100 × 100 cm square surrounded by 40-cm high walls. The movement track of each animal was monitored by a video camera placed above the arena. The EthoVision video-tracking system (Noldus, Wageningen, The Netherlands) was applied to track the immobility in mice through the measurement of the total distance moved (cm) and velocity (cm/s).

3.2. Tail-Flick test

In order to measure the acute nociception response in mice, a radiant heat, automatic tail-flick apparatus (Ugo Basile, Italy) was used. After finishing the experiment at the end of the 6-week period, mice were positioned in a restrainer, and tail-flick latency was calculated by directing a beam of light on the distal 2 cm of the tail until the mice exhibited a flick of the tail. An upper limit of 15s was set to prevent the tissue damage.

3.3. Tissue lysis and protein extraction

The caudal region of the corpus callosum was dissected from the brain. The corpus callosum was coronally transected and separated for the different experiments. The obtained tissues were suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5 mM EDTA, and protease inhibitor] and homogenized using an Ultra-Turrax T25 homogenizer (Northern Media, Nottingham). The homogenates were then incubated on ice for 30 min and centrifuged at 15,000 rpm for 15 min at 4 °C. After centrifugation, the protein extracts were recovered and stored at −70 °C until analysis.

3.4. Western blot analysis

Before the commencement of western blot analysis, the total protein concentrations in tissue homogenates were determined by bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as the standard. Firstly, the protein extracts (50 μg/lane) were electrophoretically separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% nonfat dry milk at room temperature or 37 °C for 1–2 h. The transferred proteins were probed with rabbit monoclonal antibody against Bax (1:1000 dilution), rabbit monoclonal antibody against cleaved caspase 3 (1:500 dilution), and rabbit monoclonal antibody against β-actin which was used as a loading control (1:1000 dilution). The membranes were incubated with rabbit anti-mouse IgG-HRP for 1–2 h at room temperature. The bound proteins were detected by chemiluminescence using enhanced electrochemiluminescence (ECL) reagents and subsequent autoradiography. Densitometry analysis was carried out using scanning immunoblots and quantitating protein bands using ImageJ software (National Institutes of Health, imagej.nih.gov/ij).

3.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β, IL-6, heme oxygenase-1(HO-1), and Bcl-2 were quantified using commercial ELISA kits specified for the detection of these proteins in tissue lysates. The protocols used for the measurement of the above proteins were in accordance with the manufacturer's instructions. Briefly, the tissue extracts were added to a 96-well ELISA plate and then reacted with their cognate primary antibodies and HRP-conjugated secondary antibodies. 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was used as the substrate, and the absorbance was measured at 450 nm using a microplate reader (Model 650, Bio-Rad Laboratories, USA).

3.6. The activity of glutathione peroxidase (GPx) and Caspase-12

Glutathione peroxidase (GPx) activity was calculated using the protocol developed by Paglia and Valentine [12] with minor modifications. Tissue homogenates possessed approx. 40 μg protein, were added to the mixture comprising of 100 mM Tris (pH 7.6), 0.5 mM EDTA, 1 mM DTT, 1 mM GSH, 0.2 mM NADPH and 0.4 unit/ml glutathione reductase. Upon the addition of 0.2 mM tert-butyl hydroperoxide (TBHP) (final volume 0.2 ml), the reaction was commenced, and the absorbance of NADPH (extinction coefficient 6.22 mM^{-1} cm^{-1}) was measured at 340 nm for 5 min, at 30 s intervals, at 25 °C. In order to exclude a nonspecific reduction of NADPH, the reaction was concurrently determined in the absence of TBHP. The activity of GPx was expressed as nmol NADPH oxidized/min/mg protein.

The activity of caspase-12 was calculated using Caspase-12 Fluorometric Assay Kit. In brief, 100 μl tissue homogenates were mixed with 100 μl reaction buffer (2×), and 10 μl fluorogenic substrate (ATAD-AFC) was added. The samples were then incubated for 30 min at 37 °C (in the absence of light), and fluorescence was measured in a plate reader (μQuant Bio Tek Instruments) at ex/em at 400/505 nm.

3.7. Measurement of malondialdehyde (MDA), nitric oxide (NO), and ferric reducing antioxidant power (FRAP)

MDA is produced during lipid peroxidation and can be probed with thiobarbituric acid (TBA) that can form a pink-colored adduct with maximum absorbance at 532 nm. The protocol used for the detection of MDA was similar to the method described by Schmedes et al. [13] with a minor modification. Briefly, 0.2 ml tissue homogenate, 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (adjusted to pH 3.5), 1.5 ml 0.9% TBA and 0.6 ml distilled water were vortex mixed and the mixture was placed in a water bath at 95 °C for 50 min. After cooling down to 25 °C, 1.0 ml of distilled water and 5.0 ml butanol: pyridine mixture (15:1; v/v) was added and vortex mixed. After centrifugation at 3000 rpm for 10 min, the absorbance was spectrophotometrically determined at 532 nm. The MDA concentration was computed thereby a molar extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} and values were expressed as μmol of MDA per gr tissue weight. The breakdown product of 1, 1, 3, 3-tetraethoxypropane was employed as a standard.

The production of NO was calculated by the Griess reaction assay previously described by Green et al. [14]. The concentration of NO was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthalene diamine in 5% phosphoric acid) to 100 μl of tissue homogenate. The obtained color was measured at 540 nm. The absorbance values were calculated with respect to a standard sodium nitrite curve and converted to the corresponding nitrite concentrations (μM).

The FRAP method is used for the measurement of an antioxidant capacity in which the acidic environment causes Fe^{3+} ion existing in FRAP to reduce to Fe^{2+} possessing an intense blue color, with maximum absorbance at 593 nm. The protocol of this method was in accordance with Benzie and Strain's method [15] with a slight modification. In a brief report, 50 μl of brain homogenates along with 150 μl of deionized water was added to the FRAP reagent (10 mM TPTZ and 20 mM FeCl3 in 300 mM acetate buffer, pH 3.6) leading to the increase in absorbance at 593 nm after the 5th minute of incubation period at 37 °C. FeSO4 solutions from 0.2 to 1.2 mM in 1.15% KCl were utilized for the calibration. FRAP value was expressed as μM per gr of wet tissue.

3.8. Immunohistochemical and immunofluorescence studies

After the end of behavioral tests, mice were deeply anesthetized with the intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Afterward, they were transcardially perfused with ice-cold PBS, followed by 4% paraformaldehyde (PAFA). Mice were decapitated, and the brains were removed from the skull and post-fixed with 4% paraformaldehyde for 48 h, followed by 10%, 20%, and 30% sucrose solutions, each for at least 18 h. Brain tissue was embedded in Tissue Freezing Medium (Leica, Germany), frozen at −70 °C and cut with a Leica microtome into 20-μm coronal sections. Frozen sections were utilized to detect the expressions of MBP (as a marker for the myelin), Iba-1 (as a marker for mouse microglia), and GFAP (as a marker for astrocytes). Sections were incubated for 12 h at 4 °C with primary antibodies: anti-MBP (rabbit polyclonal anti-MBP; 1:1000; Abcam), anti-Iba-1(Goat polyclonal to Iba1; 1:500; Abcam), and anti-GFAP (rabbit polyclonal to GFAP; 1:500; Abcam), Following the incubation with primary antibodies, sections were washed and incubated for 2 h at room temperature with secondary antibody: donkey Alexa Fluor 488 F(ab) anti-mouse IgG, goat Alexa Fluor 488 F(ab) anti-mouse IgG, or goat Alexa Fluor 592 F(ab) anti-mouse IgG. To visualize DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) test was used for staining DNA fragmentation to detect apoptotic cell death conforming to the protocol of the manufacturer (Roche Inc., Basel, Switzerland). Briefly, tissue sections were rinsed with PBS and treated with 3% BSA for 30 min at 37 °C before being incubated with TUNEL reaction mixture in the dark for 90 min at 37 °C. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min. The percentage of TUNEL-positive neurons was calculated by counting 500 cells in each specimen (five visual fields/specimen). The analysis of images was performed using ImageJ Software (NIH Image, imagej.nih.gov/ij). We also included the brain of mice receiving no drug solvent (DMSO) to rule out the possible effect of DMSO on the histopathology of the brains.

3.9. Copper analysis

Fresh tissues were mineralized in ultrapure HNO3 at 70 °C for 24 h. After digestion, the solution was brought to the final volume using water and filtered with a 0.2 μm pore size. The concentration of Cu in brain homogenates was determined by a Perkin-Elmer A100 flame atomic absorption spectrophotometer, using metal ion standard solutions for instrument calibration.

3.10. Statistical analysis

Data were expressed as mean ± SD. The analysis of the obtained data was carried out using the GraphPad Prism version 6.0. Comparison between groups was tested by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Values were considered statistically significant if the p-values were less than 0.05.

4. Results

4.1. The effect of rifampicin on nociception

According to Fig. 1A, six-week treatment with cuprizone did not cause significant changes nociceptive response when compared to either the control group or mice receiving rifampicin intraperitoneally. The acute nociception latency was assessed by the tail- flick test soon

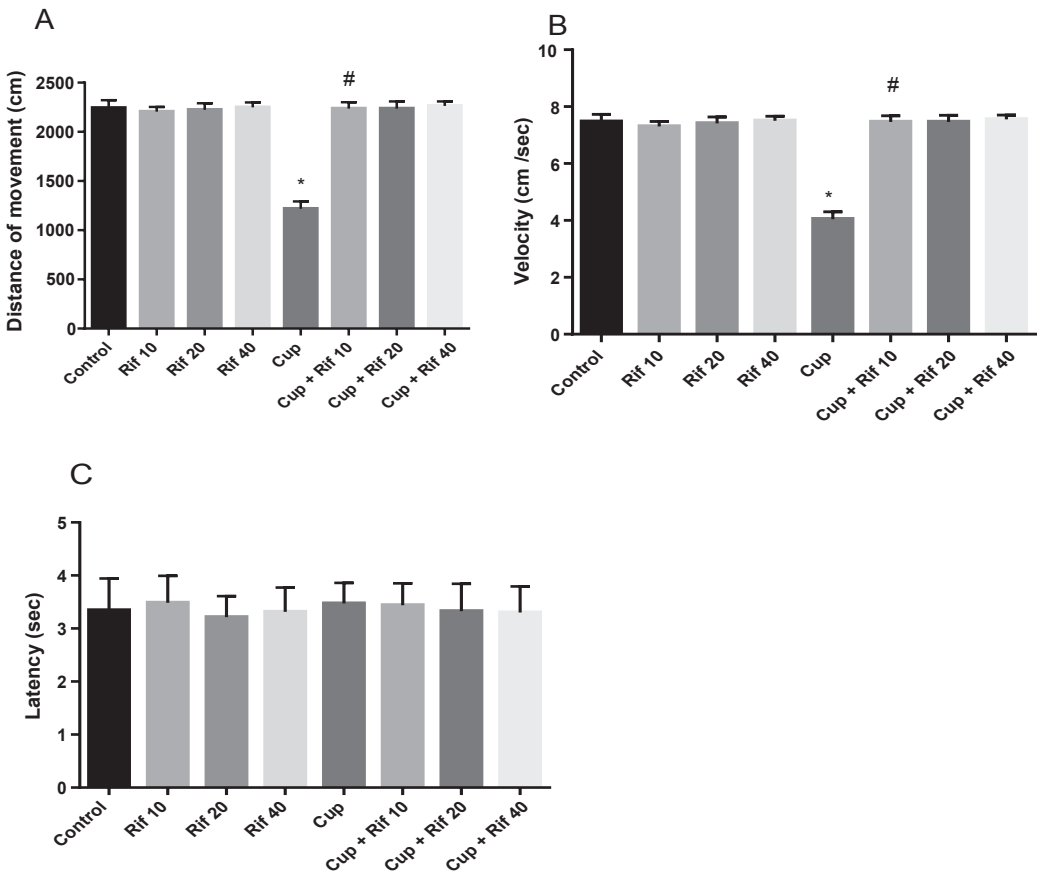

Fig. 1. The effect of different doses of rifampicin on behavior and nociception of mice treated with cuprizone. (A, B) Treatment with cuprizone lowered the length and the velocity of movement in cuprizone-treated mice while the administration of rifampin reversed the effect of cuprizone in all doses in mice treated with the drug for seven days. (C) Cuprizone treatment did not cause any significant change in nociceptive response in mice treated with either cuprizone or rifampicin for six weeks. Rif; rifampicin, Cup; cuprizone, *shows the significant change in comparison to control ($p < 0.05$). #shows the significant change in comparison to cuprizone treated ($p < 0.05$).

after the cessation of a 6-week treatment period.

4.2. The effect of rifampicin on mice movement

Feeding mice with cuprizone for five weeks caused a significant reduction ($p < 0.01$) in the distance traveled by mice (Fig. 1B). Interestingly, the administration of rifampicin significantly enhanced the movements of mice measured by the open-field apparatus ($p < 0.01$). Additionally, similar to movement measurement, a reduced velocity was indicated in mice intoxicated with cuprizone, while the administration of rifampicin significantly improved ($p < 0.01$) mice velocity by a 2-fold increment (Fig. 1C). Of note, neither was the velocity nor the distance of movement affected by rifampicin when administered in healthy mice.

4.3. Rifampicin and proinflammatory cytokines of cuprizone-treated mice

The concentrations of proinflammatory cytokines were analyzed in the homogenate of brains of mice. It was shown that levels of IL-1β were increased in brains of mice fed with cuprizone as compared with the control group. As illustrated in Fig. 2A, rifampicin significantly decreased ($p < 0.01$) the levels of IL-1β in mice treated with various doses of rifampicin in comparison to the group that received cuprizone plus DMSO. In line with this, the levels of IL-6 were increased in mice intoxicated with cuprizone whereas the intraperitoneal administration of rifampicin significantly lowered ($p < 0.01$) the concentrations of IL-6 (Fig. 2B).

4.4. Apoptosis-associated factors in mice intoxicated with cuprizone

Regarding Fig. 3A, the levels of Bcl-2 protein, an antiapoptotic factor, were significantly ($p < 0.01$) lower in mice treated with cuprizone as compared with the control group. Furthermore, the levels of Bcl-2 were statistically higher ($p < 0.01$) in mice administered with rifampicin than those mice intoxicated with cuprizone for six weeks. The activity of caspase-12 was higher in cuprizone-treated mice than the control. Notably, the administration of rifampicin significantly reduced ($p < 0.01$) the activity of this enzyme into basic levels (Fig. 3B). In accordance with Fig. 3C, western blot analysis revealed that the relative expression of Bax was increased in mice fed with cuprizone in comparison to the control mice. Likewise, rifampicin administration statistically decreased ($p < 0.01$) the relative Bax expression as the dosage of rifampicin was increased.

The relative expression of cleaved caspase-3 was ($p < 0.01$) statistically increased in mice receiving 6 weeks cuprizone when compared to the control mice. A significant reduction ($p < 0.01$) was observed in groups of mice receiving rifampicin at different doses. Semi-quantitative analysis of the above factors was performed by ImageJ software (Fig. 3D, E).

4.5. Redox-related factors in mice with cuprizone-induced demyelination

The levels of some redox-related factors have been investigated in mice treated with cuprizone to observe changes in the antioxidant capacity of the brain tissue. With regard to our results, the concentrations of HO-1 were increased in homogenates of mice with cuprizone-induced demyelination as compared to the control group ($p < 0.01$). On the other hand, treatment with rifampicin significantly ($p < 0.01$) lowered the levels of HO-1 when various doses of the drug were applied (Fig. 4A). In the same way, MDA was increased in brains of mice fed with cuprizone in comparison to the control mice receiving only DMSO ($p < 0.01$). Rifampicin administration caused a significant reduction ($p < 0.01$) in the levels of MDA in mice, and the doses of 20 mg/kg and 40 mg/kg were more efficient in MDA decline than the dose of 10 mg/

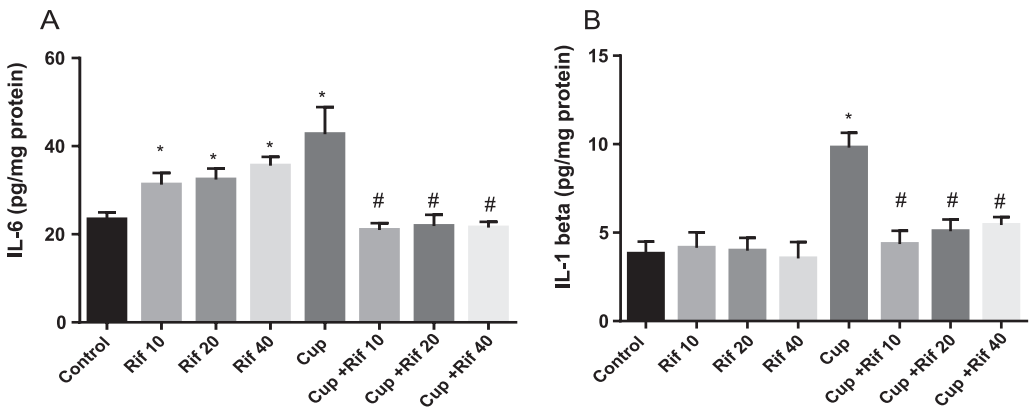

Fig. 2. The levels of proinflammatory cytokines in cuprizone-treated mice received rifampicin. (A) Treatment with cuprizone for six weeks increased the levels of IL-6 while the administration of rifampicin decreased the concentration of IL-6 in the corpus callosum of mice. (B) The levels of IL-1β is increased in mice intoxicated with cuprizone and treatment with rifampicin diminished this cytokine to the baseline level. Rif; rifampicin, Cup; cuprizone. *shows significant the change in comparison to control ($p < 0.05$). #shows significant the change in comparison to cuprizone-treated group ($p < 0.05$).

kg (Fig. 4B). The activity of GPx was decreased in homogenates of mice brains treated with cuprizone for 6 weeks when compared to mice administered with only DMSO ($p < 0.01$). Following treatment with rifampicin, the activity of GPx did not change ($p = 0.62$) even at a dose of 40 mg/kg (Fig. 4C). The levels of FRAP were diminished in the homogenate of mice brains treated with cuprizone compared to mice receiving DMSO, while intraperitoneal administration of rifampin significantly ($p < 0.01$) led to the increase in amounts of FRAP in mice receiving this drug (Fig. 4D). With respect to Fig. 4E, the level of NO was statistically ($p < 0.01$) higher in cuprizone-treated mice than the control group. Independent of drug dosage, the administration of rifampicin reduced levels ($p < 0.01$) of NO in mice receiving different concentrations of antibiotics (Fig. 4E).

4.6. Immunohistochemistry and immunofluorescence analysis of brain sections

Immunohistochemical analysis indicated that treatment with cuprizone for six weeks resulted in demyelination of corpus callosum (Fig. 5A). Treatment with rifampicin for 7 days led to the increase in remyelination compared to mice with cuprizone-induced demyelination in a dose-dependent manner. Correspondingly, the astrogliosis was increased in the corpus callosum of mice brains intoxicated with cuprizone resulted in the accumulation of astrocytes (Fig. 5A). Imaging of antibodies against the GFAP showed that rifampicin reduced the number of astrocytes in corpus callosum when compared to mice fed with cuprizone.

The degree of microgliosis was also assessed in our study as the number of microglia was enumerated via antibodies against Iba-1 in the corpus callosum of the brain sections. As shown in Fig. 5A, the number of microglia was increased in mice treated with cuprizone and the administration of rifampicin lowered the number of active microglia in a dose-dependent manner. Of note, there was no difference between the brain of mice that received DMSO as a solvent and mice receiving no treatment.

The rate of apoptosis was also evaluated using the TUNEL assay in which the number of green cells represents programmed cell death. As depicted in Fig. 5A, the number of apoptotic cells was increased in the corpus callosum of mice exposed to cuprizone when compared with mice receiving only DMSO. Rifampicin caused a significant reduction in apoptotic cells in comparison to the group treated with cuprizone in a dose-dependent manner. ImageJ software showed the relative number of cells stained with anti-MBP, anti-GFAP, anti-Iba-1, and TUNEL (Fig. 5B–E).

4.7. Copper analysis in the brain homogenate of mice

As shown in Fig. 6, the concentration of copper was decreased upon feeding mice with cuprizone during a 6-week period. According to the data, the administration of rifampicin did not change the level of copper in the brains of mice receiving cuprizone (either alone or in combination with rifampicin).

5. Discussion

It has been shown that some antibiotics possess some properties which are beyond antibacterial effects suggesting that further investigations are warranted to elucidate how they exert such beneficial roles in biological processes. The present study demonstrates that rifampicin attenuates the detrimental impacts of cuprizone on brains of C57BL/6 mice. Our results showed that intoxication with cuprizone resulted in significant reduction of mice movements at the center area of the open-field instrument. Cuprizone-fed mice tend to stay in the border area in the open field apparatus. This phenomenon stems from the deleterious effects of cuprizone on the white matter causing anxiety-like actions in mice [16] accompanied with abnormal behaviors and cognitive defects that are thought to be a direct consequence of demyelination [17]. Rifampicin was shown to diminish the behavioral deficits in mice fed with cuprizone, however; the beneficial role of the drug was independent of the dosage used for mice. The results indicated that neither rifampicin nor the cuprizone-diet did not affect the nociceptive response in the animal model which is in agreement with a study performed by Vakilzadeh et al. [18] in which they failed to show the negative impact of cuprizone on nociception.

The vast majority of studies have indicated that inflammation plays an axial role in the pathogenesis of MS [19]. The excessive inflammatory reactions can exacerbate the disease course by the production of excessive amounts of proinflammatory cytokines within the CNS and periphery [20]. The unbalanced profile of proinflammatory mediators urged researchers to seek therapeutic agents to modulate the expression of inflammatory cytokines. The present data indicate that cuprizone can elevate the expression of IL-1β and IL-6 in mice brains more than 2-fold as compared to control. Congruently, we showed that rifampicin lowered the levels of proinflammatory cytokines in the corpus callosum denoting that the suppression of inflammatory cascades paves the way to alleviate the demyelination process in mice. Not only does rifampicin exert an advantageous effect on the neurodegenerative animal model of MS in our study, but it also mitigates the severity of EAE (a neuroinflammatory model of MS in rodents) through the inhibition of pathogenic Th17 cells responses [21]. It is noteworthy that the administration of rifampicin into mice leads to the significant elevation of IL-6 in mice receiving only DMSO. Such an event was also reported by a study conducted by Ziglam et al. [22] who indicated that rifampicin increased the levels of IL-6 in LPS-induced monocytes. In the same way, Kandasamy et al. [23] demonstrated that rifampicin caused a marked increase (about 17-fold) in the expression of IL-6 in the human primary renal proximal tubular cell (HPTC) and human embryonic stem cells (hESC). They suggested that rifampicin-induced

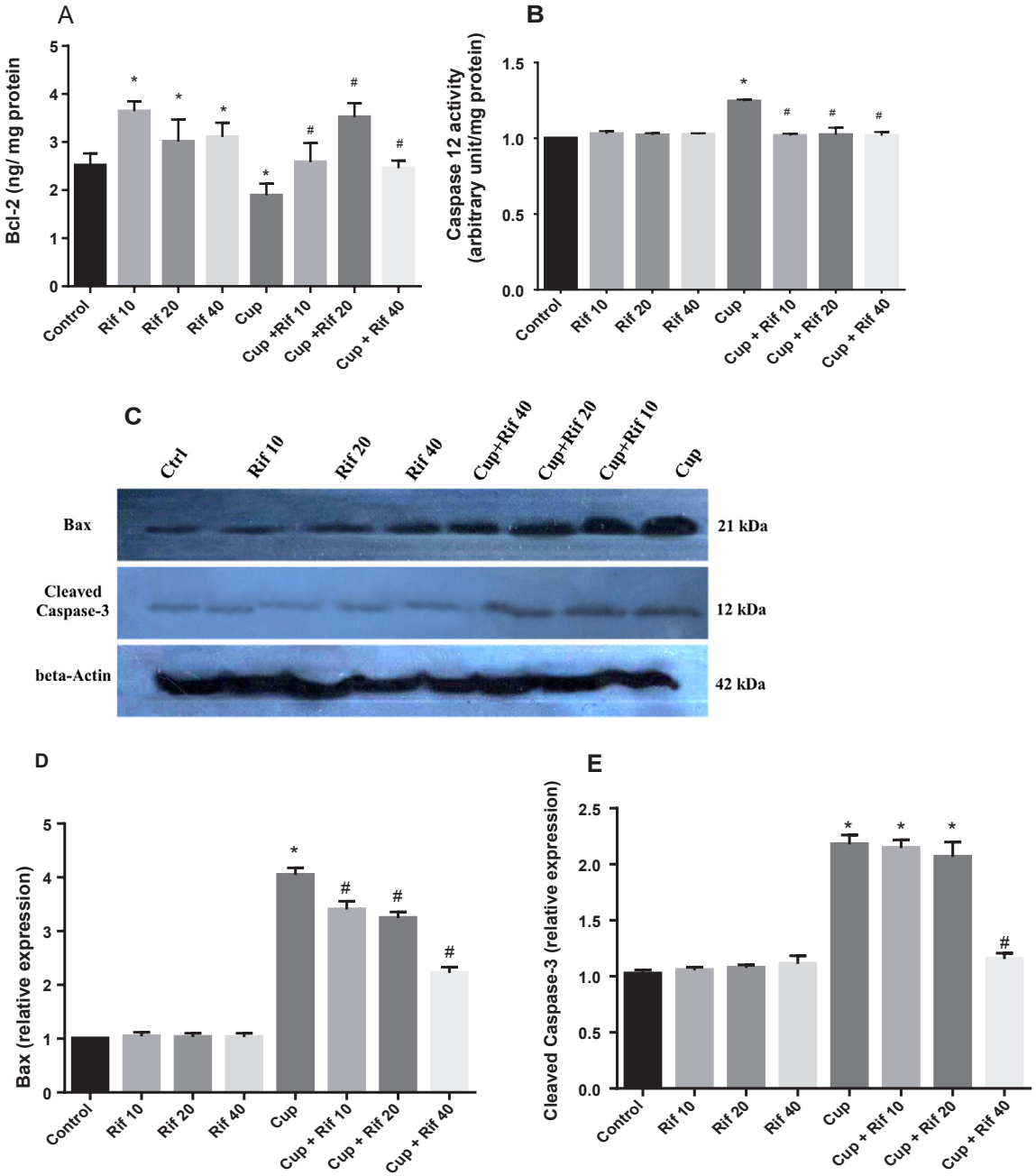

Fig. 3. Apoptosis-associated factors in response to rifampin therapy in mice treated with cuprizone. (A) The expression of Bcl-2 was decreased in response to 6-week treatment with cuprizone while the administration of rifampicin elevated the levels of Bcl-2. (B) The activity of caspase-12 was heightened in mice received cuprizone, and the treatment with rifampicin diminished the activity of caspase-12. (C) Increased expression of Bax was detected in the corpus callosum of mice treated with cuprizone for six weeks whereas rifampicin was able to decrease the expression of Bax in a dose-dependent manner. Similarly, the expression of cleaved caspase-3 was increased in mice treated with cuprizone while rifampicin reduced the rate of apoptosis through the decline in caspase-3 cleavage. Beta-actin was used as an internal control and considered the housekeeping protein. (D) Semi-quantitative expression of Bax obtained from ImageJ software. (E) Semi-quantitative expression of cleaved caspase-3 using ImageJ software. Cup; cuprizone, Rif: rifampicin, Ctrl: control. *Shows the significant change in comparison to control ($p < 0.05$). #Shows the significant change in comparison to the cuprizone-treated group ($p < 0.05$).

induction of IL6 and IL8 was dependent on transporter-mediated uptake of the drug. The mechanism by which rifampicin elevates the expression of IL-6 in normal cells is largely unknown. It seems that different cell signaling pathways are triggered when the cells (depending on cell types) are exposed to rifampicin in the absence or presence of the immune stimulants.

The antiapoptotic properties of rifampicin were also investigated in our study. Our study revealed that the anti-apoptotic factor, Bcl-2, was increased in response to 7 days treatment with rifampicin indicating that rifampicin can neutralize the apoptosis which occurs following cuprizone treatment. The decrease in cleavage of caspase-3, the ultimate episode in the apoptosis pathway, along with the reduction of Bax expression in mice receiving rifampicin provides promising evidence that rifampicin is an antiapoptotic agent mediating neuroprotection via inhibiting the expression of factors involved in programmed cell death. A study carried out by Yerramasetti and colleagues [24] showed that rifampicin down-regulated the expression of Bax, and up-regulated the expression of Bcl-2, Bcl-xL, and Flice-inhibitory protein-L (FLIPL) in Jurkat T cells. The process of apoptosis is evidently highlighted in the pathology of a cuprizone model of demyelination, as this process

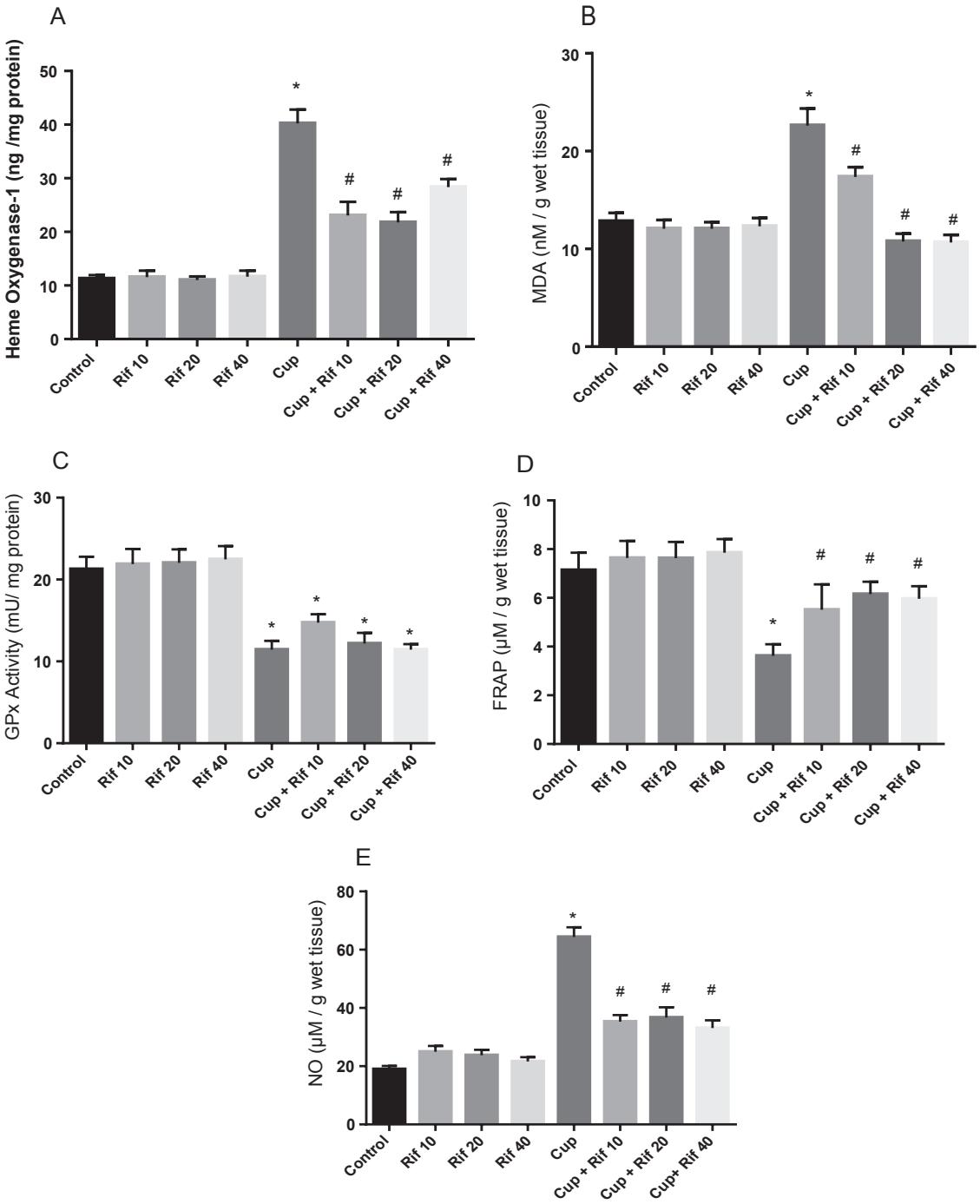

Fig. 4. The measurement of redox-related factors in homogenates of the corpus callosum. (A) Increased amounts of HO-1 was detected in mice treated with cuprizone whereas rifampicin decreased the increment occurred in cuprizone-treated mice. (B) The levels of MDA were increased in response to treatment with cuprizone while rifampicin reduced the concentration of MDA. (C) Reduced activity of GPx was shown in the brains of mice treated with cuprizone whereas rifampicin elevated the activity of the enzyme. (D) Significant reduction of FRAP was indicated cuprizone-treated mice while the administration of rifampicin significantly raised FRAP levels independent of the dosage of the drug. (E) The levels of NO were increased in mice fed with cuprizone and treatment with rifampicin diminished such levels independent of drug dosage. Cup; cuprizone, Rif: rifampicin, Ctrl: control. *Shows the significant change in comparison to control ($p < 0.05$). #Shows the significant change in comparison to the cuprizone-treated group ($p < 0.05$).

mainly affects oligodendrocyte progenitor cells (OPCs) in the corpus callosum [25]. It has been thought that endoplasmic reticulum plays a critical role in the propagation of cuprizone-induced cell death in OPCs in which cuprizone treatment causes an ATP shortage and increases ROS/RNS concentrations which can finally disrupt the proper functioning of the endoplasmic reticulum (ER) and result in apoptosis [26]. In this context, rifampicin has been indicated to exert protective cellular responses via the up-regulation of glucose-regulated protein 78 (GRP-78) which is a chaperone protein localized in the ER and plays an axial role in cell protection and survival under endoplasmic stress in response to cytotoxic agents [27].

The activation of the glucocorticoid receptors in a cuprizone-induced model of demyelination has been exhibited neuroprotective properties in which the administration of the glucocorticoid receptor agonists can alleviate inflammation of the CNS induced by cuprizone intoxication [28]. In this regard, studies indicated that rifampicin is

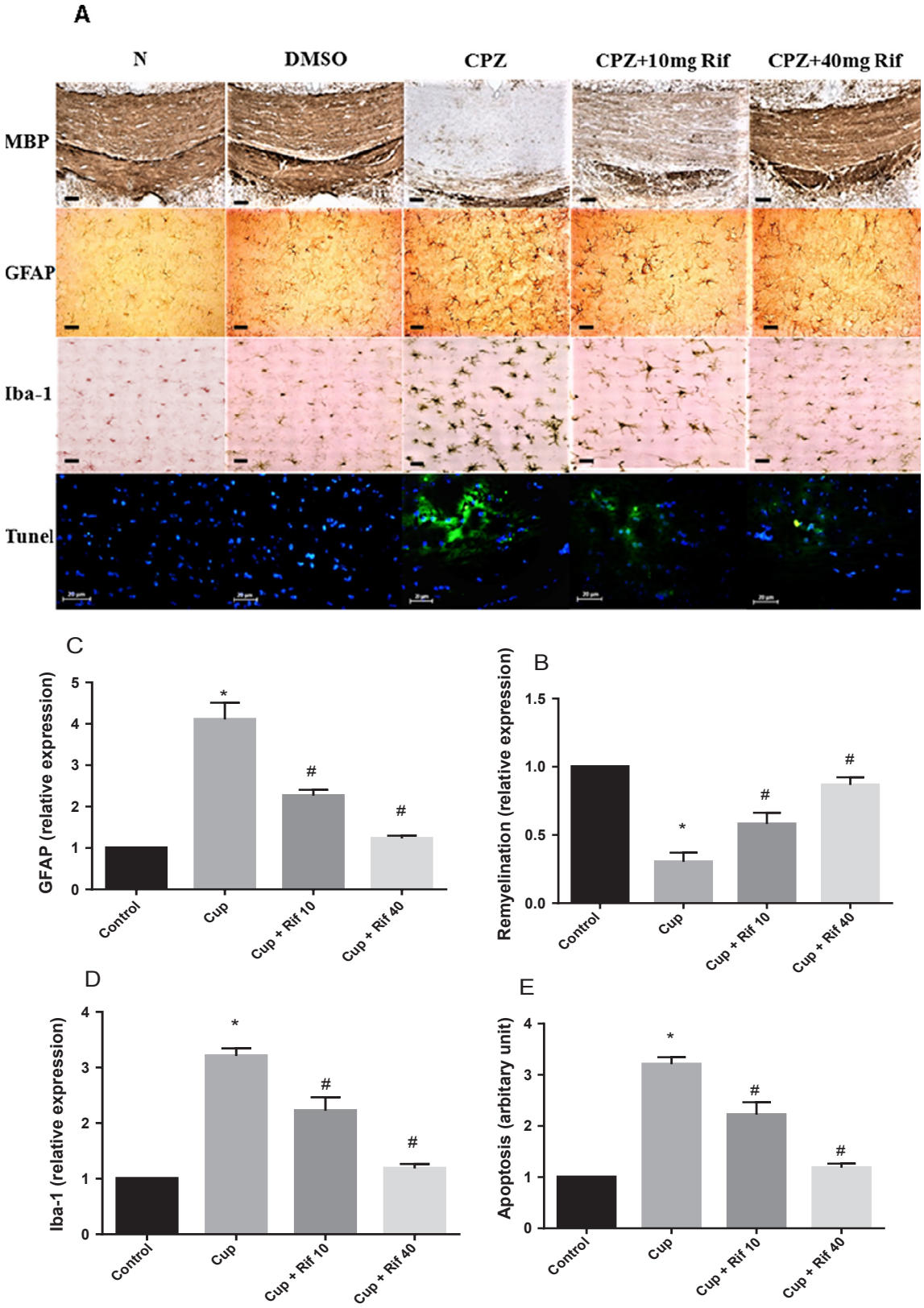

Fig. 5. The immunohistochemistry of MPP, GFAP, and Iba-1 along with immunofluorescence staining of TUNEL in brain sections in mice. (A) Immunohistochemical stating of the corpus callosum against the myelin basic protein showed that rifampicin boosted the remyelination process and reduced the astrogliosis, microgliosis, and apoptosis in a dose-dependent manner. Semi-quantitative analysis demonstrated that rifampicin enhanced (B) the remyelination process and reduced (C) astrogliosis, (D) microgliosis, and (E) apoptosis. Cup or CPZ; cuprizone, Rif; rifampicin, Ctrl; control N; normal mice that received nothing. *Shows the significant change in comparison to control ($p < 0.05$). #Shows the significant change in comparison to the cuprizone-treated group ($p < 0.05$).

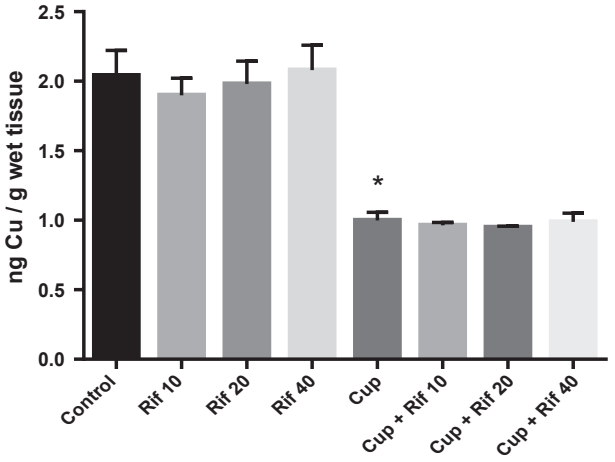

Fig. 6. The concentration of copper in the brain tissue of all groups of mice. The results show that the concentration of copper was lowered in response to treatment with cuprizone. Rifampicin did not affect chelating copper ion exerted upon feeding mice with cuprizone.

able to have agonist activities on the glucocorticoid receptors and acts as an immunosuppressive agent to decrease host-related neuronal damage by neuroinflammation [29,30].

The activity of caspase-12 was, for the first time, investigated in our study. The increment in the activity of caspase-12 in response to treatment with cuprizone, suggests that cuprizone mediates apoptosis via endoplasmic reticulum (ER) stress considered the alternative pathway for cell death. The elevation in the activity of caspase-12 was expected as it takes part in the resolution of inflammation [31].

Another pathological facet of MS is the surplus amounts of oxidative stress produced by the immune cells within the CNS of people afflicted with the disease. It has been displayed that oxidative stress is the main culprit of demyelination in the cuprizone-induced model [6]. A number of studies explored the harmful impacts of cuprizone on oligodendrocytes. It has been shown that upon intoxication with cuprizone for 5 weeks, the activity of Cu/Zn SOD is strikingly decreased that results in a deficiency of the enzyme in scavenging ROS. The accumulation of ROS in oligodendrocytes leads to triggering pathways that are destructive to the cells including elevation of lipid peroxidation, depletion of GSH contents, the decrease in levels of ferritin, and eventually cell death [32]. Interestingly, rifampicin was found to have free radical scavenging potentials with its naphthohydroquinone ring which may contribute to the function of a hydroxyl radical scavenger activities in inhibiting neurotoxicity [33].

Some redox-related factors were analyzed in this study in mice treated with cuprizone. We exhibited that the levels of HO-1 are elevated during the cuprizone treatment while the administration of rifampicin lowers the increased levels of HO-1. Since this enzyme functions as a defense system against oxidative stress and serves as a compensatory factor to maintain the redox balance within the cells [34]. It is conceivable that the heightened concentrations of HO-1 are detected following oxidative stress-induced neuronal injury as reported by Le et al. [35]. In contrast to our results, Vakilzadeh and colleagues [36] showed a reduction in the expression of HO-1 when mice were exposed to 6-weeks treatment with cuprizone. Such a discrepancy may be due to the different method used for the detection of HO-1 (ELISA vs. Western blot) and the duration of the treatment (6 weeks vs. 5 weeks). We also indicated that the increased level of MDA in response to cuprizone is reduced upon treatment with rifampicin. Of note, higher concentrations of rifampicin have a greater effect on MDA reduction. The activity of GPx and the level of FRAP were reduced in cuprizone-fed mice denoting that 6-week exposure to cuprizone can weaken the antioxidant capacity of cells. It has been disclosed in our study that cuprizone is able to increase levels of NO, in parallel to ROS generation, to implement cytotoxicity against the neurons and myelin sheath. Surprisingly, rifampicin was able to empower the antioxidant capacity of the mice brains fed with cuprizone and made them resistant to the devastating effects of this toxin without creating harmful events by itself.

The imaging technique was applied as corroborative evidence for rifampicin efficacy in the promotion of remyelination when used in various doses. Our results indicate that remyelination is enhanced as the dosage of rifampicin is increased. The results obtained in this research were consistent with a study conducted by Ma et al. [21] in which rifampicin stimulated remyelination in a dose-dependent manner. We showed that the concentration of copper was lowered in response to the intoxication of mice fed with cuprizone. The data displayed that rifampicin was not able to interfere with the absorbance of cuprizone in the gastrointestinal (GI) tract of mice suggesting that rifampicin plays a direct effect on the brain and does not make a perturbation in the uptake of cuprizone from the GI tract. The fidelity of rifampicin was reinforced by its actions in reducing the number of astrocytes and microglia which are upregulated during cuprizone treatment. Rifampicin successfully modulated the expressions of GFAP and Iba-1 to control the number of active astrocytes and microglia, respectively. Besides, the TUNEL assay verified the anti-apoptotic effect of rifampicin which was described earlier in ELISA and western blot experiments.

Regarding the results obtained from immunohistochemistry analysis, similar to previous studies, substantial demyelination occurred well before the 5th week of cuprizone treatment due to the excessive amounts of inflammatory mediators and neurotoxic agents within the brain of mice. However, the degree of the remyelination process was remarkably higher as compared with the corresponding research. One of the reasons for the increased remyelination rate in comparison to other studies is that rifampicin was administrated when the intrinsic remyelination process was initiated at the beginning of the 6th week of cuprizone treatment. It has been spotlighted that the inherent remyelination process becomes apparent at the 6th weeks of cuprizone treatment [37,38]. Hence, it seems that rifampicin boosts remyelination instead of inhibiting demyelination process, and has no interference with the uptake of cuprizone. The effect of rifampicin on the suppression of demyelination will be demonstrated if rifampicin is administrated as a co-treatment with cuprizone during a 5-week cuprizone feeding. All in all, further studies are warranted to illuminate whether rifampicin can alone impede the destruction of myelin sheath when concomitantly administrated with cuprizone.

6. Conclusion

It seems that rifampicin can be considered as a complementary treatment for moderating MS. The evidence shows that rifampicin has been useful to both neuroinflammatory (EAE) and neurodegenerative (cuprizone-induced demyelination) animal models.

Acknowledgment

The present research was supported by the collaboration between the University of Tehran and Professor Keyvani's laboratory. We thank Dr. Sreeram Ramagopalan for language editing.

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