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Araştırma Makalesi

PROTECTIVE MECHANISM OF Morus nigra ON CARBON TETRACHLORIDE INDUCED BRAIN DAMAGE IN RATS

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ABSTRACT

Morus nigra (MN) is a common fruit used all over the world. Hepatic encephalopathy (HE) is a dangerous neuropsychiatric complication of both acute and chronic liver failure, and is the most common cause of death in patients with end-stage liver disease. To our knowledge, this is the first study investigated the efficiencies of MN on the treatment of HE on cirrhotic rat liver model induced by carbon tetrachloride (CCI_4). In this study it has been used superoxide dismutase (SOD) and catalase (CAT) activities assays, 8-hydroxydeoxyguanosine (8-OHdG) immunohistochemistry and Haematoxylin-Eosin (H&E) staining in the brain, as well as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in liver tissue. H&E staining was used to examine hippocampal morphology alterations. 8-OHdG staining was employed to measure the expression of apoptotic-related proteins. With this aim, 42 Sprague-Dawley male rats were divided into six groups: control, HE control (1 mL/kg CCI_4/twice a week, intraperitoneal), 125 mg/kg MN, 250 mg/kg MN, CCI4+125 mg/kg MN and CCI_4+250 mg/kg MN groups. CCI_4 caused a significant increase in serum enzyme levels in rat livers, compared with control. On the other hand, MN treatment restored the biochemical parameters significantly in a dose-dependent manner. 8-OHdG activity was significantly increased in CCI_4 group. However, these increases were significantly decreased by MN treatment. While intraperitoneal administration of MN significantly reduced oxidative stress, prevented apoptosis and caused an increase in the antioxidant defence mechanism activity in brain compared to the control group. Brains of rats treated with MN showed less DNA damage than treated groups with CCI_4. As a result of this study showed that MN has a neuroprotective effect because of its anti-inflammatory, anti-apoptotic, antioxidant properties.

Keywords: *Morus nigra, hepatic encephalopathy, carbon tetrachloride, neurotoxicity, antioxidant enzymes*

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SIÇANLARDA KARBON TERAKLORÜR İLE UYARILMIŞ BEYİN HASARINDA Morus nigra' NIN KORUYUCU MEKANİZMASI

ÖZET

Morus nigra (MN) tüm dünyada yaygın olarak kullanılan bir meyvedir. Hepatik ensefalopati (HE) hem akut hem de kronik karaciğer yetmezliğinde tehlikeli bir nöropsikiyatrik komplikasyondur ve son dönem karaciğer hastalığı olan hastalarda en sık görülen ölüm nedenidir. Bilgimize göre bu, karbon tetraklorür (CCI_4) ile indüklenen sirozlu sıçan karaciğeri modeli üzerinde HE tedavisinde MN'nin etkilerinin araştırıldığı ilk çalışmadır. Bu çalışmada, karaciğer dokusunda aspartat aminotransferaz (AST) ve alanin aminotransferaz (ALT) seviyelerinin yanısıra beyin dokusunda 8-hidroksideoksiguanozin (8-OHdG) imünohistokimya ve Hematoksilen-eozin (H&E) boyaması, superoksid dismutaz (SOD) ve katalaz (CAT) aktivite testleri kullanıldı. H&E boyaması, hipokampal morfoloji değişikliklerini incelemek için kullanılmıştır. 8-OHdG boyaması apoptotik ilişkili proteinlerin ekspresyonunu ölçmek için kullanıldı. Bu amaçla 42 Spraque-Dawley erkek sıçan 6 gruba ayrıldı: kontrol, HE kontrol (1 mL/kg CCI_4/haftada 2, intraperitonal), 125 mg/kg MN, 250 mg/kg MN, CCI_4+125 mg/kg MN ve CCI_4+250 mg/kg MN grupları. CCI_4 sıçan karaciğerlerinde kontrol ile karşılaştırıldığında serum enzim seviyelerinde belirgin bir artışa sebep oldu. Diğer taraftan MN uygulaması biyokimyasal parametreleri doza bağımlı olarak önemli ölçüde düzeltti. 8-OHdG aktivitesi CCI_4 grubunda belirgin olarak arttı. Ancak MN tedavisi ile bu artışlar önemli ölçüde azaldı. MN'nin intraperitoneal olarak uygulanması, kontrol grubuna kıyasla oksidatif stresi önemli ölçüde azaltırken, apoptozisi önledi ve beyindeki antioksidan savunma mekanizması aktivitesinde artışa neden oldu. MN ile tedavi edilen sıçan beyinleri CCI_4 ile tedavi edilen sıçanlara göre daha az DNA hasarı gösterdi. Bu çalışmanın sonucu MN'nin anti-inflamatuar, antiapoptotik, antioksidan özelliklerinden dolayı nöroprotektif etkiye sahip olduğunu gösterdi.

Anahtar kelimeler: *Morus nigra, hepatik ensephalopati, karbon tetraklorür, nörotoksisite, antioksidan enzimler*

INTRODUCTION

Medically, metabolic brain disease is defined as acquired or innate metabolic disease that affects the way the brain functions. Hepatic encephalopathy (HE) is associated with hepatic insufficiency and is characterized by a plethora of central nervous system abnormalities and different experimental models have been used to study the HE and liver failure, being the most common the one that uses carbon tetrachloride (CCI_4, a potent hepatotoxic drug that causes cell damage leading to the death of the liver cells and then tissue fibrosis (1). HE occurs due to a combination of distinct pathophysiological mechanisms such as inflammation, oxidative stress, impaired blood–brain barrier (BBB) permeability, neurotoxins, impaired energy metabolism of the brain (2, 3, 4). Animal models can be used to distinguish components of the processes associated with damage to the brain in response to CCl4 exposure (5, 6, 7). The molecular mechanism underlying the toxic effects of CCl4 involves lipid peroxidation (LPO), mediated by the free radicals that are generated during its metabolism (5). Lavrentiadou and collegues have mentioned that CCI_4 intoxication induces oxidative stress-mediated elevation of t-PA and PAI-1 activities in rat brain. Elevated t-PA proteolytic activity may resultas a response to increased tissue remodeling requirements in the injured area. The common Greek herbs, oregano and rosemary, when administered in a long-term mode can moderate these effects, thus suggesting their potential to protect the CNS from oxidative damage (8).

Moraceae is a family of flowering plants that comprises about 40 genera and over 1,000 species. Mulberry (*Morus* sp.) has been domesticated over thousands of years and has been adapted to a wide area of tropical, subtropical and temperate zones of the World. Morus nigra (MN) has been used in folk medicine as an analgesic, diuretic, antitussive, sedative, anxiolytic and hypotensive, in addition to its uses in the treatment of a variety of ailments, including inflammatory disorders (9). These actions were reported by the presence of flavonoids, which have powerful antioxidants that are associated with their medicinal properties, including antidiabetic activity (10).Souza and colleagues have also mentioned that Mn extract shows strong antibacterial and antioxidant activities (11). Antioxidants act by removing and scavenging reactive oxygen species (ROS), and its precursors, and binding to metal ions necessary for the catalysis of ROS generation (12). Many reports attest to the fact that increased consumption of medical and aromatic plants is associated with protection against various disorders such as cancer, cardiovascular, hepatic and neurological diseases (13, 14, 15). Moreover, recent reports postulate that pro-inflammatory and oxidative stress pathways may be crucial mechanisms involved in the pathogenesis of this disease (16).

In this context, the present work evaluated the neuroprotective effect of MN extract in CCl4 induced HE in rats.

MATERIAL AND METHODS

Plant Material and Extract Preparation

MN was collected from Gümüşhane and Erzurum provinces in Turkey during the summer of 2013. The plant was identified and authenticated by experts from Botanical Department (Atatürk University, Erzurum, TURKEY). Identified samples were air-dried. 100 g MN was weighted and splitted with blender device for each treatment. Then, samples were divided into two parts. In the first part, 100 mL-purified water was added to the splitted sample. It was extracted at the

room temperature for a night. Furthermore, it was filtered and the operation was repeated. After alcohol was added to the second part of splitted black mulberry cells, extracted, and it was filtered and removed by an evaporator dissolver. After the extracts were combined, they were filtered into filter paper, and then the filtrates were taken to ballons, and frozen in deepfreeze. The frozen extracts were lyophilized under the pressure of 50 mm-Hg until it dried in lyophilizer. Extracts were incubated at 4 ºC until use.

Animals and Experimental Design

Forty two adult male Spraque-Dawley rats (weighing 290–320 g) obtained from Medical Experimental Application and Research Center, Atatürk University, were used. Animals were housed inside polycarbonate cages in an air-conditioned room (22ºC± 2 ºC) with 12-h light–dark cycle. Standard rat feed and water were provided at libitum. All procedures were performed in conformity with the Institutional Ethical Committee for Animal Care and Use at Atatürk University (protocol number: 36643897-188/13) and the Guide for the Care and Use of Laboratory Animals.

In this experiment, the rats were randomly divided into six groups, each of whom has seven rats. Group 1 (C): Control rats; rats were intraperitoneally injected with physiological saline solution, Group 2 (ºC): Rats were intraperitoneally injected with 30 % ºC mixed with soybean oil (1 mL/kg body weight), Group 3 (low dose MN): Rats treated with MN 125 mg/kg bw, Group 4 (high dose MN): Rats treated with MN 250 mg/kg bw, Group 5 (ºC+low dose MN): Rats with hepatic encephalopathy treated with MN-125 mg/kg bw, Group 6 (CCl4+High dose MN): Rats with HE treated with MN-250 mg/kg bw. The extracts of MN was administered intraperitoneally daily for eight weeks.

Histopathological examinations and assessments

After anesthetization and getting blood samples from the tail of the rats and their brain was removed for macroscopic, histopathological, and biochemical analyses. Brain sections were fixed in 10% formalin and then embedded in paraffin to form blocks. The samples were then serially-sectioned (5-µm thick) using a Leica RM2135 microtome (Leica, Berlin, Germany), mounted on glass slides and then stained using H&E solution. The pathological changes were assessed and photographed with a light microscope (Nicon Eclipse E600, Olympus) at 10 and 20 magnifications and photographed.

Immunohistochemical examination

Immunohistochemical staining for 8-OHdG proteins were performed by an automated method on the VENTANA BenchMark GX System (Ventana Medical Systems) with an ultraView Universal DAB Detection Kit on 4-µ sections from a representative block in each rat. After deparaffinization to water, the antigenic determinant sites for 8-OHdG, were unmasked in citrate buffer with steam for 60 min. The primary antibody 8-OHdG (Santa Cruz sc-393871) was used at a dilution of 1:300 for 32 min at 37°C. The slides were then incubated with the diluted antibody, followed by application of Ultraview Universal DAB detection kit (Ventana Medical Systems). DAB was used as a chromogen and hematoxylin as a counterstain. The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of primary antibody on tissue from the same animal.

Biochemical analysis

Estimation of serum biochemical

parameters.

Blood samples from animals were collected in gel-activated tubes for the assessment of specific liver markers. The gel-activated tubes were allowed to clot, then centrifuged at 4000 × g for 10 min at 4°C. The serum samples were collected for measuring liver markers, ALT and AST. (commercial kits on a Beckman Coulter AU5811 device, Japan).

CAT assay

CAT activity was assayed at 25 °C using a method based on the decomposition of H_2O_2 (16). Diluted homogenate (1:40; 5 µL) was added to 720 µL of 30 mM H_2O_2, in 10 mM potassium phosphate solution; the reaction was monitored at 240 nm. Under these conditions, the decomposition of H_2O_2 by the CAT contained in the samples exhibits first-order reaction kinetics according to the equation k = 2.3/t log(Ao/A), where k is the first-order reaction rate constant, t is the time period over which the decrease in H_2O_2 as a result of CAT activity was measured (15s), and Ao and A are the optical densities at 0 and 15 s, respectively. The results were expressed as k/mg protein.

SOD assay

SOD activity was assayed using a previously reported method (7). A competitive inhibition assay was performed using the xanthine-xanthine oxidase system to reduce NBT. In a final volume of 166 µL, the reaction mixture contained the following: 0.122 mM EDTA, 30.6 µM NBT, 0.122 mM xanthine, 0.006% bovine serum albumin and 49 mM sodium carbonate. The liver homogenate (1:50 dilution; 33 µL) was added to the reaction mixture, followed by 30 µL of a xanthine oxidase solution to a final concentration of 2.5 U/L. This reaction mixture was then incubated at room temperature for 30 min. The reaction was stopped with 66 µL of 0.8 mM cupric chloride, and the optical density was read at 560 nm. Complete NBT reduction (100%) was obtained in a tube in which the sample was replaced by distilled water. The amount of protein that inhibited NBT reduction by 50% was defined as one unit of SOD activity. The results were expressed as U/mg protein.

Statistical Analysis

Data recording and analysis was performed on "SPSS 20.0 for Windows" (SPSS Inc., IL, USA) software. Descriptive data were expressed as mean±standard deviation .Compatibility with normal distribution of SOD and CAT levels and serum AST, and ALT results was assessed using the Kolmogorov-Smirnov test. Since all results were normally distributed, comparisons of them among the groups was performed using parametric one-way ANOVA, while degree of significance of differences between groups was determined using the post hoc LSD test. Correlations between results were assessed using Pearson correlation analysis. $P<0.05$ was regarded as significant.

RESULT

Effect of MN extracts on serum biochemical markers after CCI_4 damage

The administration of CCI_4 significantly increased serum levels of AST and ALT (Table 1). MN (125, 250 mg/kg) caused significant ($P < 0.05$, $P < 0.01$) dose-dependent reductions in CCI_4-elevated levels of ALT and ALT. In addition, treatment of animals with MN alone did not show any significant change in liver enzymes compared with the control group (Table 1).

Table 1. Effects of CCI4 and MN on the activity of rat serum ALT and AST.

Group	ALT(U/L)	ALT(U/L)
Control	54.76±7.86	192.19±9.78
LD-MN	50.85±7.58	183.21±15.03
HD-MN	48.72±8.55	171.32±13.09
CCl_4	193.67±16.04*	320.78±10.01*
CCl_4+LD-MN	97.21±23.08	228.51±10.98
CCl_4+HD-MN	78.10±32.12	203.12±14.45

Data are expressed as mean ± standard deviation *P<0.05, according to ANOVA followed by the Tukey post hoc test.

Effects of MN extracts on antioxidant enzymes

CCI_4-induced damage in the brain decreased the activity of antioxidant enzymes ($p < 0.0001$) (Table 2). High dose MN (HD-MN) extract provided the strongest protection in the presence of SOD and CAT; however, low dose MN (LD-MN) extract also showed protective effects for SOD and CAT (Table 2).

Table 2. Effects of Mn extracts on antioxidant enzyme activity in the brain of rats with CCl4-induced damage.

Group	SOD	CAT
Control	58.17±1.25	0.011±0.002
LD-Mn	62.71±1.02	0.013±0.001
HD-Mn	63.82±1.23	0.012±0.003
CCl_4	32.01±1.75*	0.003±0.002*
CCl_4+LD-Mn	45.93±1.53	0.008±0.005
CCl_4+HD-Mn	49.71±1.68	0.009±0.004

CCl4 effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each antioxidant activity measured: *$p < 0.0001$ vs all groups (in SOD); *$p < 0.0001$ vs all groups. Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD

Histopathological results

Sections from the brain of control group exhibited normal neuronal structure. Neurons retained their shape and normal cellularity with obvious nuclei (Figure 1A). Sections from the CCI_4-treated rats showed marked neuronal degeneration; neurons decreased in number and had indistinct boundaries. The sections also exhibited irregular damaged cells and cytoplasmic shrinkage. There was evidence of pyknotic nuclei and chromatin condensation. Necrosis and perineuronal vacuolation were observed (Figure 1B). The Brain tissue of rats coadministered MN and CCI_4 showed few pyknotic nuclei (Figure 1C). Higher doses of Mn (250 mg/kg) protected neurons against the degenerative alterations caused by rotenone (Figure 1D).

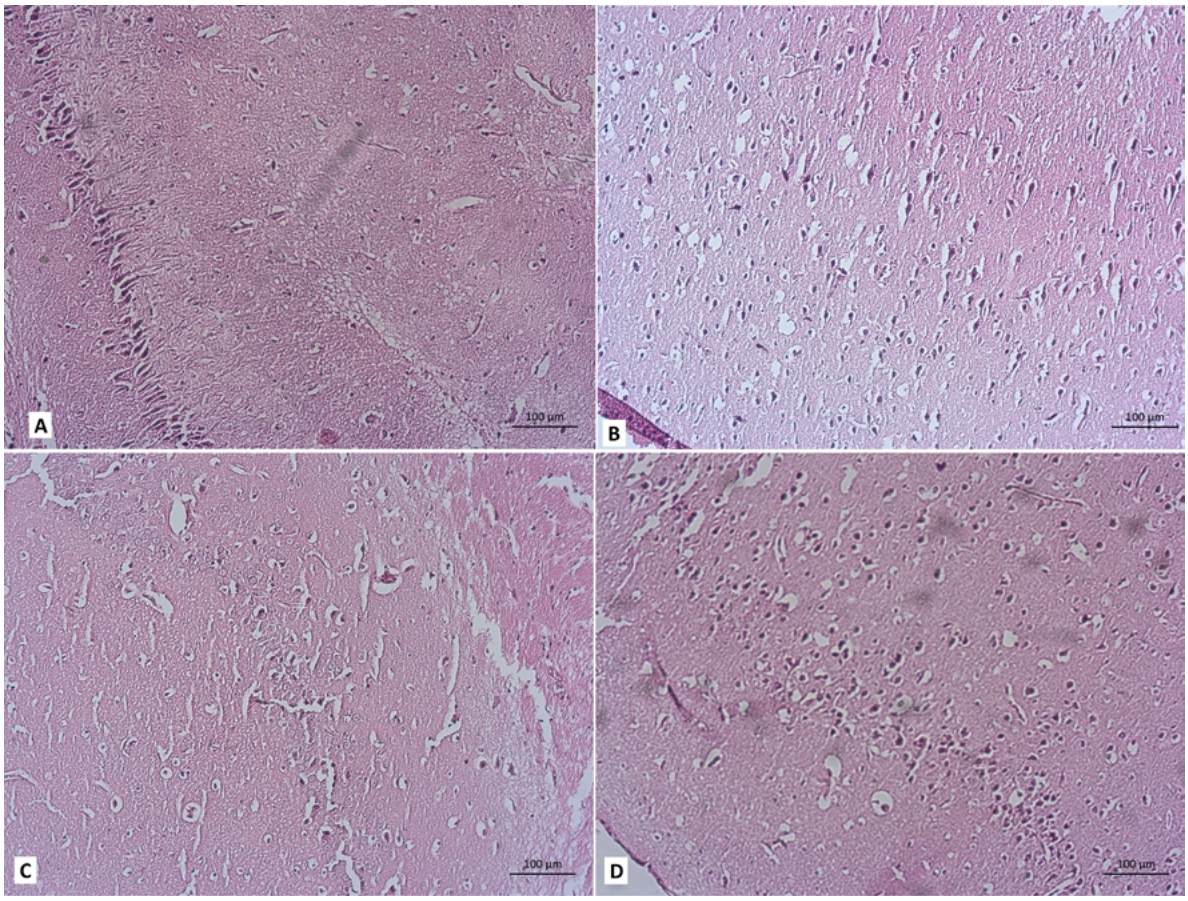

Figure 1. Representative light microphotographs of H&E stained sections from rats brain treated with physiological saline solution, CCI_4, or CCI_4 + MN.(A) Control group: neurons with surrounding supporting cells with normal nuclei that showed dispersed chromatin and prominent nucleoli. The cytoplasm of these cells was basophilic. (B) CCI_4 : extensive neuronal damage, degeneration, loss of neurons and surrounding cells. Neurons appear smaller and shrunken with slight vacuolation of neuropil. Pyknotic darkly stained nuclei, apoptotic cells and cytoplasmic vacuolations. (C) CCI_4+MN-125: nearly normal morphological appearance of with fewer apoptotic cells and cytoplasmic vacuolation (D): CCI_4+MN-250: Mild pyknotic darkly stained nuclei, apoptotic cells, and cytoplasmic vacuolations (magnification at 40×).

Immunohistochemistry

Sections from the brain of Normal saline -treated rats did not show immunopositive cells (Figure 2A). However, the CCI_4-only group displayed extensive immunopositive cells of 8-OhdG (Figure 2B). On the other hand, 8-OHdG positive cells were reduced after cotreatment with Mn and CCI_4 compared with the CCI_4-only treated group (Figure 2C, 2D).

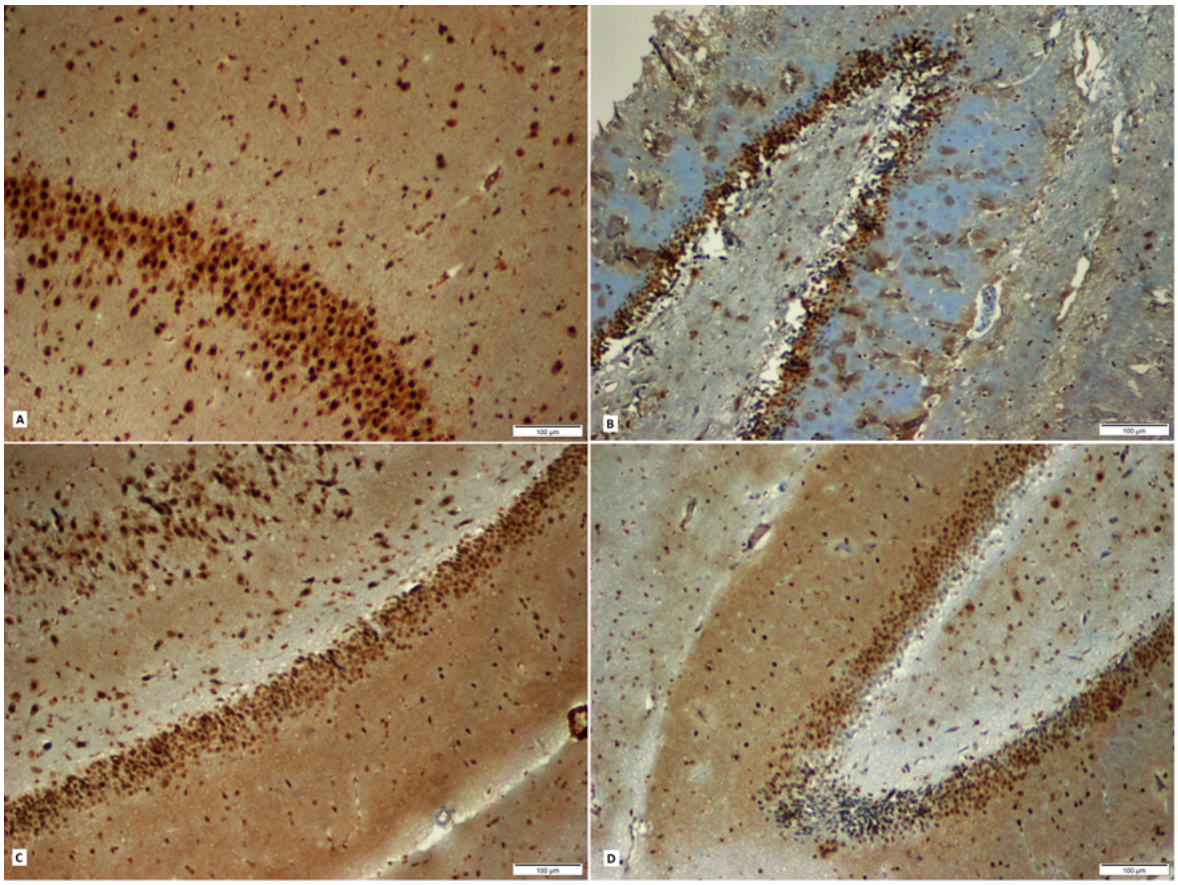

Figure 2. IHC staining showing the protective effect of MN against CCI_4-induced neurodegenetive disease. High dose Mn treatment significantly reduced 8-hydroxy-2′-deoxyguanosine (8-OHdG) expression. (A) control group: no immunopositive reaction. (Normal saline); (B) CCI_4 treated group: Large number of immunopositive cells compared to control. (C) Low dose MN + CCI_4: gradual increase of immunostaining dependent on the dose compared to CCI_4 treated group. and (D) High dose MN: Less number of immunopositive cells compared with the CCI_4 treated group (magnification at 40×).

DISSCUSION

Administration of CCI_4 to rats induces release of hepatic enzymes, hepatocyte necrosis and production of LPO products, such as malondialdehyde (MDA) (18,19). Indeed, in our experiments, administration of CCI_4 increased blood serum ALT and AST activities, in agreement with previous results (7). The recorded rise in liver injury biomarkers in CCI_4-treated rats indicates the substantial hepatic damage produced by CCI_4.

In the present study, CCI_4 induced a marked oxidative stress in the brain. The brain tissue is highly vulnerable to oxidative stress (20). Oxidative damage of neuronal cells has been causatively implicated in multiple neurodegenerative diseases (21).

Over the years CCI_4 has been used as an excellent model for studying experimentally induced neurotoxicity in murinemodels. CCI_4 an organic industrial solvent used in industry is a vigorous carcinogenic agent that may create lung, liver, kidney and nervous system dysfunction (22, 23). CCI_4 has been demonstrated to play an important role in various neurodegenerative diseases. Our data demonstrated that oxidative stress mediates cytotoxicity induced by the restraint stress in rat's brain. The brain is one of the fetal structures that extremely prone to oxidative stress. MN has been indicated to reverse

brain damage, hyperglycemia and oxidative stress. We also observed that a significant reduction in antioxidant enzyme activities in the chronic stress in rats happened. The neurotoxicity is due to a reaction with the superoxide anion, which results in the formation of peroxynitrite capable to nitrate and oxidize proteins, lipids, and nucleic acids (24). Results from the present experiments indicate that MN increases antioxidant enzyme activity in different brain regions. High dose MN appeared to be more effective compared to low dose, thus indicating a potential of these diets to provide protection against liver damage. However, none of them could restore enzymatic activities to those of the control (untreated) animals.

A series of experimental studies have demonstrated that MN could reduce the production of ROS and ROS-induced apoptosis (10, 14). Chemo-preventive agents, such as phenolic antioxidants, dithiol ethiones and isothiocyanates, have been shown to selectively induce the activation of antioxidant enzymes through the Nrf2 pathway (25, 26). CCI_4 induced apoptosis that was confirmed by increased 8-OHdG expression in the brain tissue. The results revealed that oxidative stress-induced brain tissues of CCI_4-treated rats evidenced a remarkable elevation in 8-OHdG (marker of DNA oxidation). While CCI_4 increased the number of apoptotic cells, this increase was prevented in CCI_4+125 mg/kg MN and 250 mg/kg MN groups. The mechanism of neurotoxicity is thought to be related to the increased oxidative stress by the toxin. In the present study, we found that MN has marked antioxidant action in the experimetal model of brain injury by decreasing the apoptosis. It has been reported that 8-OHdG, which is the most commonly analyzed biomarker of oxidative damage to DNA, showed higher levels in the brains of AD patients than in subjects with normal aging (27). Results from our study also indicate that 8-OHdG positive cells were reduced after co-treatment with MN and CCI_4 compared with the CCI_4-only treated group. However, further experimental studies are required in order to determine the minimum effective concentration. Nevertheless, the results of the present study indicate a possible pro-oxidant action of MN at higher concentrations. It has been suggested that the learning Impairment manifested by cirrhotics with hepatic encephalopathy is restored post-transplant (28).

The lipid solubility of CCI_4 enables it to cross cell membranes, and when it is systemically administered, it is distributed and deposited mainly in the liver and brain. The time course of the elimination of CCI_4 appears to be governed by the blood perfusion rate and the lipid content of the tissue (29). We showed that CCI_4 significantly decreased the activity of the antioxidant enzymes CAT and SOD in the brain, likely due to protein inactivation by free radicals and these findings are in line with those reported previously (30,31). The antioxidant capacity of flavonoids is well known, and they can act as antioxidants in chemical systems due to their extensive conjugated π-electron systems that facilitate the donation of electrons from the hydroxyl moieties to the oxidizing radical (32). Some studies indicated that black mulberry leaf is a rich source of phenolic compounds which provides a potential antioxidant activity (33,34).

The present study demonstrated that CCI_4 treatment elicited toxicological consequences in our animal models. On the other hand, the potential properties of MN extract in restoring these toxicological

consequences were properly investigated. In conclusion, the present study suggests that CCI_4 intoxication induces oxidative stress in rat brain. MN extract when administered in a long-term mode can moderate these effects, thus suggesting their potential to protect the brain from oxidative damage.

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