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The Effect of Melatonin on Behavioral, Molecular, and Histopathological Changes in Cuprizone Model of Demyelination

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Abstract Multiple sclerosis (MS) is an autoimmune, demyelinating disease of the central nervous system. The protective effects of melatonin (MLT) on various neurodegenerative diseases, including MS, have been suggested. In the present study, we examined the effect of MLT on demyelination, apoptosis, inflammation, and behavioral dysfunctions in the cuprizone toxic model of demyelination. C57BL/6J mice were fed a chaw containing 0.2 % cuprizone for 5 weeks and received two doses of MLT (50 and 100 mg/kg) intraperitoneally for the last 7 days of cuprizone diet. Administration of MLT improved motor behavior deficits induced by cuprizone diet. MLT dose-dependently decreased the mean number of apoptotic cells via decreasing caspase-3 and Bax as well as increasing Bcl-2 levels. In addition, MLT significantly enhanced nuclear factor-κB activation and decreased heme oxygenase-1 level. However, MLT had no effect on interleukin-6 and myelin protein production. Our data revealed that MLT improved neurological deficits and enhanced cell survival but was not able to initiate myelin production in the cuprizone model of demyelination. These findings may be important for the design of potential MLT therapy in demyelinating disorders, such as MS.

Keywords Pineal gland · Remyelination · Cell death · Corpus callosum · Neuroinflammation

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Abbreviations

MLT	Melatonin
CNS	Central nervous system
ECL	Electrochemiluminescence
EAE	Experimental autoimmune encephalomyelitis
H&E	Hematoxylin and eosin
HO	Heme oxygenase
IL-6	Interleukin-6
LFB	Luxol Fast Blue
MS	Multiple sclerosis
NFκB	Nuclear factor-κB
PFA	Paraformaldehyde
BBB	Blood brain barrier
PNS	Peripheral nervous system
PLP	Proteolipid protein
PMP-22	Peripheral myelin protein 22
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, which leads to myelin sheet breakdown and neuronal loss [1]. Glial cell death, especially oligodendrocytes, has a critical role in the pathophysiology of MS [2]. According to different types of animal model of MS, feeding with cuprizone, a copper chelating agent, can induce reversible demyelination in different parts of the brain, especially in the corpus callosum [3]. This toxic model of demyelination is characterized by oligodendrocyte degeneration via copper-dependent mitochondrial enzymes inhibition [4, 5]. Besides oligodendrocyte death, astrogliosis as well as activated microglia and macrophages affect demyelination progression by secreting pro-inflammatory cytokines [5]. Blood brain barrier (BBB) remains intact during cuprizone treatment and therefore local damage occurs without contribution of immune system and lymphocytes infiltration [6].

Melatonin (MLT; N-acetyl-5-methoxytryptamine) is an endogenous hormone, which is mainly secreted by the pineal gland [7]. Besides its well-known functions, such as controlling the circadian rhythm, it has been suggested that MLT has neuroprotective properties by modulation of oxidative stress, neuroinflammation, mitochondrial homeostasis, and apoptosis [8]. This hormone has a high lipophilicity with excellent biomembrane permeability that enables it to freely cross the BBB. After exogenous administration, MLT was found in high concentrations in the brain [9]. In addition, MLT can reach subcellular compartments through its two types of receptors: *i*) G-protein couple receptors (MT1, MT2) that can initiate intracellular signal transduction cascades [10, 11] and *ii*) quinone reductase enzyme family (MT3), which has binding site for selective agonists and antagonists as well as similar affinity to MLT and its precursor, N-acetylserotonin [12].

MLT neuroprotective effects have been reported by several experimental investigations. It has been suggested that MLT has an anti-apoptotic effect in some neurodegenerative diseases, like Alzheimer's disease, stroke, and Huntington's disease [13]. Furthermore, MLT can reduce acute and chronic inflammation by decreasing pro-inflammatory cytokines and modulating serum inflammatory parameters [13, 14]. MLT administration increased fibers myelination and oligodendrocytes maturation by decreasing white matter inflammation after neonatal stroke [9] and exhibited an anti-nociceptive action in an animal model of acute pain [15]. In experimental autoimmune encephalomyelitis (EAE) model, MLT ameliorates the severity of paralysis accompanied by less spinal cord infiltration of inflammatory cells [16]. Furthermore, increases in serum melatonin concentrations have been suggested as a possible mechanism underlying the effectiveness of some drugs in treatment of MS [17]. The aim of this study was to examine the effects of MLT on behavioral changes as well as molecular and histopathological alterations in cuprizone toxic model of demyelination.

Materials and Methods

Animals and Demyelination

Male C57BL/6 mice with body weight ranging between 20 and 25 g (8 to 9 weeks old) were obtained from Pasteur Institute, Tehran, Iran. They were maintained on a 12-h light/dark cycle in room temperature between 20 and 22 °C and had free access to food and water. To induce demyelination, mice were fed with a diet containing 0.2 % cuprizone mixed into ground standard rodent chow for 5 weeks. Control animals were fed normal powdered chow. All animal manipulations were carried out according to the Ethical Committee for the use and care of laboratory animals of Tehran University of Medical Sciences and Shefa Neuroscience Research Center.

Experimental Design

Mice were divided randomly into 4 groups: (*i*) control group which received normal powdered chow for 5 weeks with intraperitoneal (i.p.) injection of ethanol 1 % (diluted in saline) for 7 days, (*ii*) cuprizone group that were fed powdered chow mixed with 0.2 % cuprizone for 5 weeks, (*iii*) MLT group which were divided into 2 separated subgroups, treated with 50 or 100 mg/kg of MLT (i.p.) injected in midday for the last 7 days of 5 weeks cuprizone feeding, and (*iv*) healthy group which were divided into 2 subgroups which received 2 doses of MLT (50 and 100 mg/kg) separately in midday for 7 days. All mice from different groups were evaluated via behavioral, molecular, and histopathological tests.

Behavioral Experiments

Open-Field Test

To evaluate the effect of MLT on motor impairment, all groups were assessed by open-field test at the end of the 5th week of experiment. Animals (each group, $n=7$) were placed in an open-field box, and locomotion was tracked over a 3-min period. The EthoVision tracking system (Noldus Information Technology, Wageningen, The Netherlands) was used to evaluate motor function by measuring the total distance moved (cm) and velocity (cm/s); [18, 19].

Tail-Flick Test

A standardized tail-flick apparatus (tail-flick Unit 7360, Ugo Basile, Italy) with a radiant heat source connected to an automatic timer was used to assess acute nociception response. After the end of the 5th week, each animal was placed in a restrainer and the tail-flick latency was measured by focusing a beam of light on the distal 2 cm of the tail until the animal

exhibited a flick of the tail. Cut-off time (10 s) was used to minimize tissue damage [20].

Western Blot Analysis

For Western blot analysis, the caudal region of the corpus callosum was dissected from the brain. Samples were homogenized and centrifuged at 12000 rpm for 20 min with lysis buffer containing a complete protease inhibitor cocktail. Bradford's method was used to determine protein concentrations [21]. Bovin serum albumin was considered for generating standard plot. In the next step, total proteins were separated in 12 % SDS PAGE gels electrophoretically and after transferring to polyvinylidene-fluoride membranes, probed with specific antibodies. Immunoreactive peptides were detected by chemiluminescence using enhanced electrochemiluminescence (ECL) reagents (Amersham Bioscience) and subsequent autoradiography. Results were quantified by densitometric scan of films, and data analysis was done using ImageJ, measuring integrated density of bands after background subtraction. Nuclear and cytoplasmic proteins were isolated as described [22].

Histopathological Studies

Ketamine (i.p, 50 mg/kg) and xylazine (4 mg/kg) were used for mice anesthesia after the 5th week of experiment. Thereafter, mice were perfused with 4 % paraformaldehyde (PFA). Brains were dissected, post-fixed in 4 % PFA, and embedded in paraffin for 24 h. Finally, coronal sections were obtained in 6-μm thickness from 1.58–2.30 mm from the bregma).

For assessing myelination, Luxol Fast Blue (LFB) staining was used for paraffin sections. Briefly, after anhydration, brain sections were incubated with 0.1 % LFB solution at 60 °C during the night. The slides were differentiated in lithium carbonate solution for 30 s and then in 70 % ethyl alcohol for another 30 s after rinsing with 95 %, 70 % ethanol and distilled water. Following another wash with distilled water, the sections were counterstained with 0.1 % cresyl fast violet for 10 s. Thereafter, sections were washed with distilled water, dehydrated in a graded series of alcohols, cleared in xylene, and finally mounted [23]. The ventral body of the corpus callosum was observed with an Olympus light microscope (BX51, Olympus, Japan) and photographed with an Olympus digital camera (Olympus, Japan) linked to a microscope. ImageJ software was used to evaluate the extent of demyelination based on the ratio of blue to pink fibers in the corpus callosum as the percentage of volume fraction in damage tissue/total area for 16 sections in each animal (n=4). Furthermore, hematoxylin and eosin (H&E) staining was performed to evaluate inflammation.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) test was used for staining DNA fragmentation using an in situ Cell Death Detection Kit (Roche, Germany) to evaluate DNA fragmentation. Three 6-μm-thick tissue sections were dewaxed from each block, dehydrated by 60 °C heating, with a following washing in xylene. Thereafter, sections were rehydrated through washing with diluted alcohol. After being washed with 10 mM Tris-HCl (pH 7.6), incubation in methanol was performed for tissue sections containing 0.3 % H_2O_2 for 10 min to inhibit endogenous peroxidase activity. They were then treated with proteinase K (Roche, 20 μg/ml in Tris buffer) at 37 °C for 30 min, incubated in TUNEL reaction mixture (450 μl of label solution and 50 μl of enzyme solution) at 37 °C for 60 min and in POD solution for 30 min. The color reaction was developed in 3-3'–diaminobenzidine (DAB, Roche; 0.5 μl DAB and 1.5 μl peroxide buffer) for 5–10 min with cresyl violet counterstaining. The percentage of TUNEL-positive neurons was calculated by counting 500 cells in each specimen (five visual fields/specimen). Sections were examined on an Olympus microscope (CX31, Tokyo, Japan) with a ×40 objective lens and images were captured using a digital camera (Olympus, Japan); [24, 25].

Drugs

MLT, ketamine, and xylazine were purchased from Sigma-Aldrich. Antibodies directed against β-actin, nuclear factor-κB (NFκB), caspase-3, and Bax and Bcl-2 were obtained from Cell Signaling Technology. Lamin-B2, interlukein-6 (IL-6), proteolipid protein (PLP), and peripheral myelin protein 22 (PMP-22) antibodies were purchased from Santa Cruz Biotechnology. Heme oxygenase-1 (HO-1) antibody was obtained from ABCAM. ECL kit was obtained from Amersham Bioscience. Ethanol and cresyl fast violet were purchased from Merck, and Cell Death Detection Kit was obtained from Roche. MLT was initially dissolved in ethanol and diluted to a concentration of 1 % ethanol.

Statistical Analysis

For all experiments, data were analyzed using GraphPad Prism 5. Comparison between groups was tested by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. Statistical significances were gained when p <0.05.

Results

The Effect of MLT on Animal Movement

In the current study, cuprizone and MLT groups were fed a diet containing 0.2 % cuprizone for 5 weeks. Two doses of MLT were injected (i.p.) for the last 7 days of cuprizone feeding. The day after, animals were investigated by open-field test for distance moved and movement velocity. Cuprizone feeding significantly decreased distance moved and movement velocity compared to

the control group (n=6). Two different concentrations of MLT dose-dependently enhanced distance moved and movement velocity in mice affected by cuprizone alone compared to the cuprizone group (n=12, Fig. 1 a, b). MLT alone did not affect these parameters in the healthy group (n=6).

The Effect of MLT on Nociception

The analgesic effect of two doses of MLT on cuprizone model was assessed by tail-flick test. The acute nociception latency was evaluated the day after 5 weeks of cuprizone diet. As Fig. 2 shows, cuprizone feeding did not change the nociception latency (n=6) but application of MLT at both 50 and 100 mg/kg in these mice dose-dependently and significantly increased the nociception latency (n=6). However, MLT alone in healthy animals had no significant effect on nociception response (n=6).

The Effect of MLT on Apoptotic Markers

Cuprizone diet increased Bax to Bcl-2 ratio, a key factor in the regulation of apoptosis, indicating that apoptosis enhanced via Bax upregulation (n=6). MLT administration dose-dependently diminished this ratio, confirming MLT anti-apoptotic effect in cuprizone model of demyelination (n=12). MLT alone did not show any changes in Bax/Bcl-2 ratio in the healthy group (n=6, Fig. 3a). After 5 weeks of cuprizone diet, cleaved active caspase-3 level significantly increased, indicating its involvement in oligodendrocytes apoptosis (n=6). MLT administration dose-dependently diminished caspase-3 (n=12, Fig. 3b). Injection of MLT did not alter caspase-3 level in the healthy group (n=6). Based on TUNEL assay, the mean number of TUNEL-positive cells significantly increased after 5 weeks of cuprizone diet (n=6). MLT treatment only at 100 mg/kg significantly diminished the mean number of TUNEL-positive cells in the medial region of the corpus callosum compared to the cuprizone group (n=6, Fig. 3c).

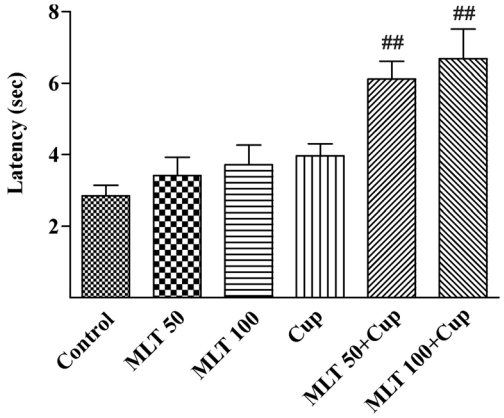

Fig. 2 The effect of cuprizone (*CUP*) application and intraperitoneal injection of different concentrations of melatonin (*MLT*) on the tail-flick test. Administration of CUP did not induce any significant effect on nociceptive responses compared to control mice. MLT also did not alter nociceptive responses in control mice. However, application of MLT at 50 and 100 mg/Kg during the last 7 days of a 5-week CUP treatment significantly increased the nociception latency compared to other animal groups (##p 0.01). Data represent the mean±S.E.M.

The Effect of MLT on NFκB, IL-6, and HO-1 Level

A significant increase in nucleus NFκB levels occurred after 5 weeks of cuprizone diet compared to the control group (n=6). Application of MLT, however, did not reduce NFκB levels. MLT administration dose-dependently increased NFκB level (n=12, Fig. 4a). MLT did not affect NFκB levels in the healthy mice group (n=6). The level of IL-6 increased after 5 weeks of cuprizone diet compared to control mice. However, application of MLT for the last 7 days of cuprizone feeding did not change IL-6 level compared to the cuprizone group (n=12). Furthermore, the healthy group receiving MLT did not show any differences in IL-6 level compared to control mice (n=6, Fig. 4b). HO-1 value significantly decreased following 5 weeks of cuprizone diet. Additionally, administration of MLT dose-dependently diminished its amount compared to the cuprizone group. MLT alone did not affect HO-1 level in healthy mice when compared to the control group (n=6, Fig. 4c). In H&E staining,

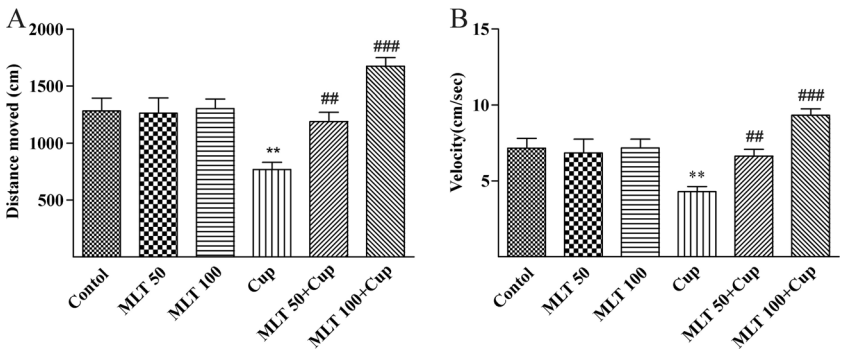

Fig. 1 Effect of two different concentrations of melatonin (*MLT*) on behavior of cuprizone (*CUP*)-treated mice at the open-field test. Mice treated by MLT at 50 mg/Kg (##p 0.01) and 100 mg/Kg ($^{###}p$ 0.001) during the last 7 days of a 5-week CUP treatment traveled longer distances (**a**) at higher velocities (**b**). Data represent the mean±S.E.M.

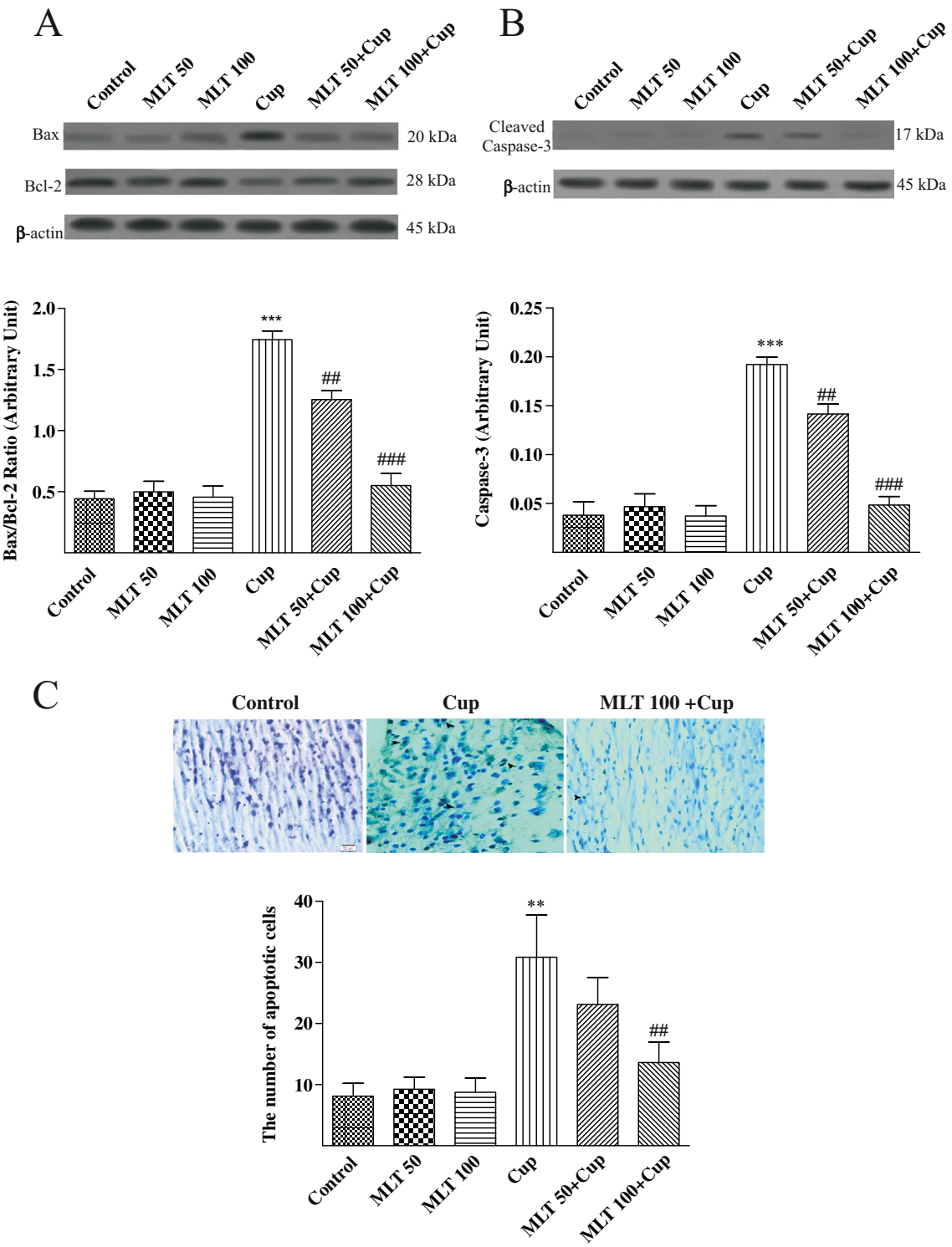

Fig. 3 Effect of two different concentrations of melatonin (*MLT*) on apoptotic markers in cuprizone (*CUP*)-treated mice. Using Western blot technique, the effects of MLT at different concentrations (50 and 100 mg/Kg) on Bax/Bcl-2 ratio (**a**) and caspase-3 activation (**b**) were tested in mice treated with CUP for 5 weeks. The densities of Bax, Bcl-2 (**a**), and caspase-3 (**b**) bands were measured, and their ratio to β-actin was calculated. ***p 0.001 represents difference from control group, ##p 0.01 and ###p 0.001 represent differences from the cuprizone group. Values indicate mean±S.E.M. The number of apoptotic cells was evaluated by TUNEL test (**c**). TUNEL-positive cells were significantly increased in the cuprizone group compared to control mice (**p 0.01). MLT at a concentration of 100 mg/kg significantly decreased the mean number of TUNEL-positive cells compared to the cuprizone group (##p 0.01). Data are given as mean±S.E.M. *Scale bar* equals 50 μm (×40)

hematopoietic cell infiltration as an indicator of tissue inflammation was assessed. Our data showed a significant increase in hematopoietic cells after 5 weeks of cuprizone diet compared to the control group. MLT injection did not significantly affect hematopoietic cell infiltration compared to the cuprizone group (n=6, Fig. 4d).

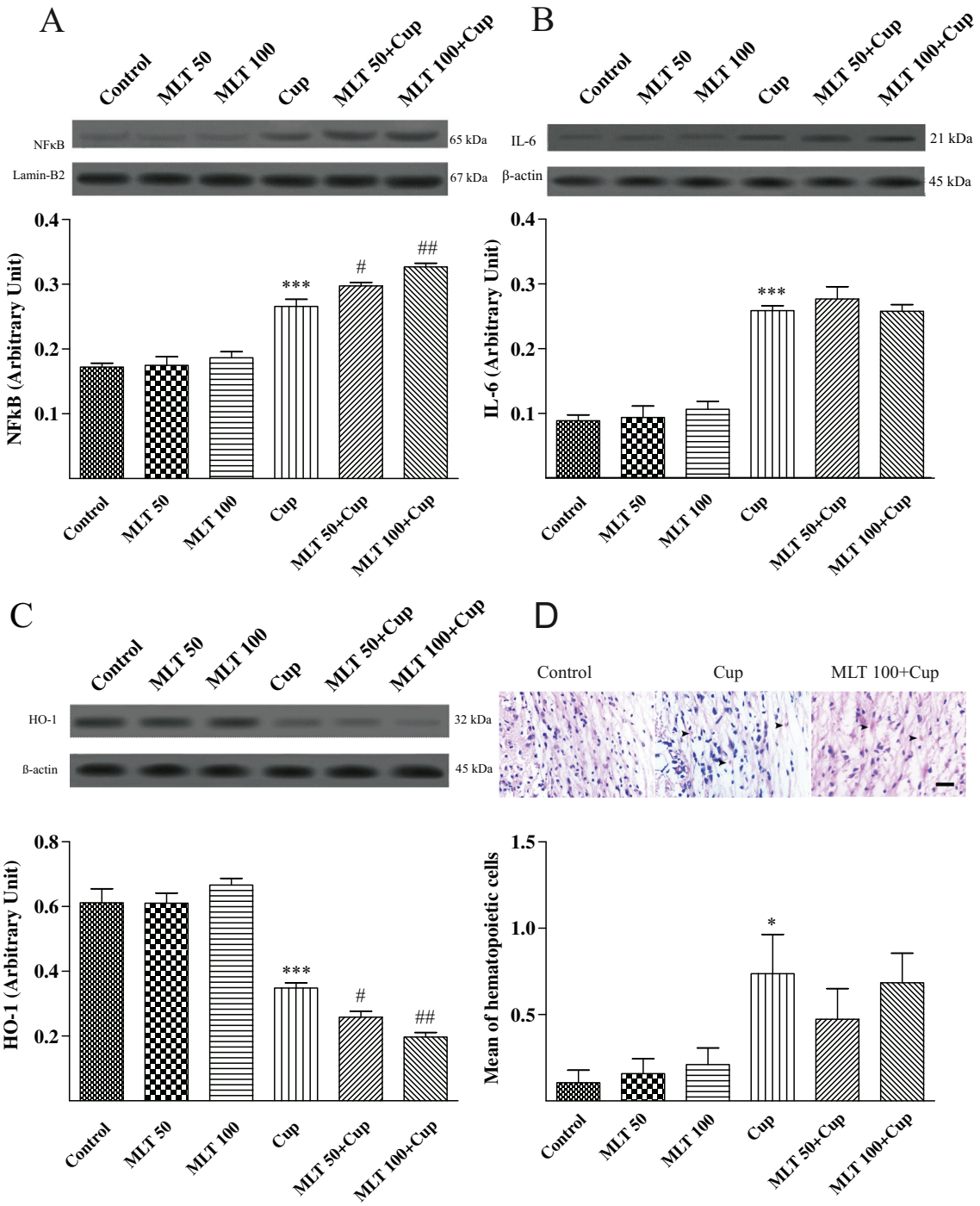

Fig. 4 Effect of two different concentrations of melatonin (*MLT*) on NFκB, IL-6, and HO-1 levels in cuprizone-treated mice. Western blot technique was used to study the effects of MLT at different concentrations (50 and 100 mg/Kg) on NFκB (**a**), IL-6 (**b**), and HO-1 (**c**) levels in the corpus callosum in mice treated with BUC. The densities of NFκB (**a**), IL-6 (**b**), and HO-1 (**c**) bands were measured, and their ratios to Lamin-B2 for NFκB and β-actin for IL-6 and HO-1 were calculated. ***p 0.001 represents difference from control group, #p 0.05 and ##p 0.01 represent difference from the cuprizone group. The effect of MLT on the number of hematopoietic cells in the corpus callosum was evaluated by hematoxylin and eosin staining (**d**). The mean number of hematopoietic cells was significantly increased in the cuprizone group compared to control mice ($^{*}p$ 0.05). There was no statistically significant difference among MLT groups and cuprizone in the number of hematopoietic cells. Data represent mean±S.E.M. *Scale bar* equals to 50 μm (×40)

The Effect of MLT on Myelination

PLP (central nervous system (CNS)-specific myelin protein) and PMP-22 are myelin sheet proteins that were considered as indicators of myelination process. According to Fig. 5, cuprizone diet significantly decreased the amount of these proteins after application for 5 weeks (n=6, Fig. 5a, b). However, neither MLT administration in cuprizone-treated animals (n=12) nor MLT injection to healthy animals (n=6) could increase myelin protein levels. In order to show demyelination

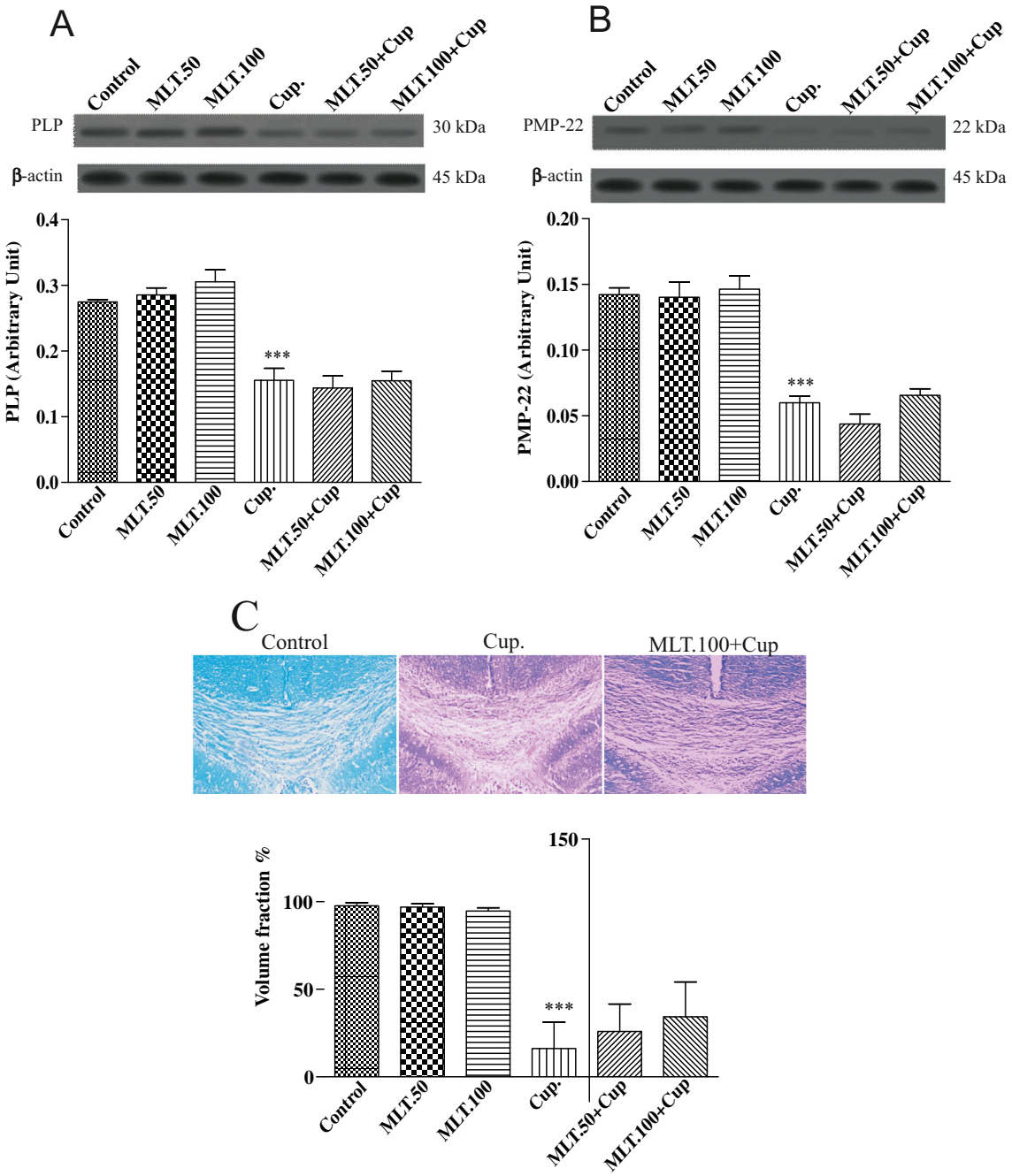

Fig. 5 Effect of two different concentrations of melatonin (*MLT*) on myelin proteins in cuprizone (*CUP*)-treated mice. Using Western blot analysis, the effects of MLT at different concentrations (50 and 100 mg/Kg) on myelin proteins PLP (**a**) and PMP-22 (**b**) were tested in mice treated with CUP. The densities of PLP (**a**) and PMP-22 (**b**) bands were measured, and their ratios to β-actin were calculated. ***p 0.001 represents difference from the control group. The severity of demyelination in the corpus callosum was evaluated with LFB staining (**c**). The *blue areas* show intact region (in the control group) whereas the *pink areas* show demyelinating areas. There is no statistically significant difference among MLT groups and cuprizone in the severity of demyelination. Percentage in volume fraction in damage tissue/total area was measured for 16 sections in each animal. *Scale bar* 50 μm (×10)

severity, LFB staining was used to evaluate percentage of volume fraction. Cuprizone diet significantly decreased myelin protein levels, compared to control mice (n=6). MLT did not increase this level compared to the cuprizone group (n= 12, Fig. 5c).

Discussion

In the current study, the effects of MLT on behavioral deficits, apoptosis, and inflammation induced by 5 weeks of cuprizone diet were investigated. A significant demyelination was detectable after 3 weeks of cuprizone treatment along with primary oligodendroglia apoptosis and microglia activation while maximum demyelination was observed after 5 weeks [4]. Our results that showed 5 weeks cuprizone feeding declined distance moved and velocity in mice is in agreement with another study in which cuprizone induced motor skills and behavioral dysfunctions [26]. MLT injection improved distance moved and velocity in cuprizone-fed animals. It has been previously shown that higher MLT plasma concentration was accompanied by improvement of general condition of MS patients [27], and MLT reduced the severity of paralysis in the EAE model of demyelination [16]. Protective effect of MLT on motor recovery was also reported after spinal cord injury in mice [17].

In this study, cuprizone diet did not change pain threshold examined by Tail-flick test. This indicates that somatosensory neurons were probably not affected by cuprizone toxic effect. However, MLT administration raised nociception latency in cuprizone-treated animals, suggesting anti-nociceptive effect of MLT in this model. Anti-nociceptive effect of MLT has been shown at spinal and supraspinal levels [28]. This effect lasts over hours due to MLT short half-life [29]. However, our results showed long acting anti-nociceptive effect of MLT, suggesting that MLT might probably stimulated other pathways that have long lasting anti-nociceptive effect [30]. Furthermore, it has been shown that some analgesics are not effective in the reduction of nociceptive responses evoked in normal condition but it is effective in a situation of central sensitization [31].

This finding was in line with other studies indicating that MLT prevented induction of TUNEL-positive cells in neuronal tissues [32, 33]. Three proteins that play major roles in regulating apoptosis were investigated: Bcl-2 as well as Bax from B cell lymphoma related family proteins, known to have a major role in the regulation of mitochondrial membrane permeability [34], and caspase-3, well-known as a downstream executioner caspase of caspase-dependent apoptotic cascades [35]. Using various experimental models, several studies revealed that MLT could block caspase-3 activation [36], increase Bcl-2 anti-apoptotic protein level [37, 38], and diminish Bax pro-apoptotic protein [37]. Our data revealed an increase of caspase-3 level after 5 weeks of cuprizone diet. Activated caspase-3 expressed in oligodendrocytes plays a crucial role in cell death in the EAE model of demyelination [39]. MLT was previously shown to be a powerful antioxidant that decreases caspase-3 activity [36]. In our study, administration of MLT prevented induction of apoptotic cells and reduced caspase-3, suggesting MLT ability to inhibit apoptosis via intrinsic pathway. Furthermore, MLT decreased Bax level versus a significant increase in Bcl-2 amount, which results in Bax/Bcl-2 ratio regulation in neuronal tissues [37, 38]. Our results revealed that cuprizone feeding increased Bax/Bcl-2 ratio, an effect that was counteracted by application of MLT. This suggests the ability of MLT to regulate cuprizone-induced apoptosis via the mitochondrial-dependent intrinsic pathway [39]. Besides its direct effect on apoptotic and anti-apoptotic markers, it has been reported that MLT might decrease apoptosis via regulating the function of mitochondrial complex, which leads to reduction of electron leakage and generation of free radicals [40].

There are several studies which report activation of astrocyte NFκB leads to oligodendrocyte death under cuprizone diet [2, 41]. In this study, nuclear NFκB levels were evaluated using Western blot technique. Activation of astrocytes in different conditions, such as cuprizone feeding, leads to NFκB upregulation and oligodendrocyte death [34, 42]. In the current study, NFκB level increased after cuprizone diet and administration of MLT further increased its level. Antithetical results were reported on MLT effect on inflammatory processes. While some investigations point to anti-inflammatory effect of MLT [8, 14, 43], some other studies reported stimulating pro-inflammatory cytokines release [44, 45] and activating NFκB translocation [1] after administration of MLT. Several different factors, such as the stage of inflammation, pathological conditions in which several cell parameters are changed, and different levels of action or targets of MLT, may determine anti- or pro-inflammatory effects of MLT [46–48].

It has been suggested that activation of NFκB is critical for cell survival after MLT application [46]. HO-1 is one of the isoforms of HO, a microsomal enzyme, which is expressed at low levels under basal conditions but can be produced in high amounts in oxidative stress and acts as an antioxidant and anti-inflammatory agent [49]. Cuprizone diet, in our study, diminished HO-1 level, and MLT further decreased its amount. It has been reported that activation of NFκB can suppress the expression of antioxidant genes by indirectly downregulating HO-1 [50].

MLT has a potent protective effect on white matter by reduction of microglial activation and subsequently enhancing oligodendroglial maturation and myelination [9, 14]. In this study, the amount of myelin proteins (both PLP and PMP-22) diminished after cuprizone feeding. MLT administration, however, did not change the myelin protein levels and myelin volume fractions. This suggests that MLT does not have an

impact on demyelination/remyelination process in the cuprizone model. Evidence from patients suffering from MS suggests that demyelination alone is insufficient to explain the loss of neurologic functions, including motor function impairments, associated with the disease. A combination of oligodendrogliopathy and axonopathy has been predicted as the main pathophysiology of neurologic dysfunction in MS [51]. Experimental investigations also revealed that demyelination is necessary but insufficient for axon injury and loss of neurologic function in an animal model of MS [52]. Axonal pathology, as a possible consequence of oligodendrocyte inflammation, is not limited to demyelinated lesions but extends into normal-appearing white matter and contributes to neurological deficits [53]. However, it should be noted that in contrast to our study, application of MLT at 5 mg/kg reversed cuprizone-induced demyelination in adult male mice [54].

In the present study, MLT was ineffective in triggering myelination but improve motor function and enhanced cell survival in cuprizone toxic model of demyelination. Since oligodendrocytes are highly vulnerable in an inflammatory context [51, 52], MLT may enhance oligodendrocyte survival by reducing white matter inflammation [9, 14] and subsequently promotes axonal regeneration and improves neurological deficits [55] in demyelinating disorders, such as MS.

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References

1. Lassmann H, Brück W, Lucchinetti CF (2007) The immunopathology of multiple sclerosis: an overview. Brain Pathol 17(2):210–8. doi:10.1111/j.1750-3639.2007.00064.x
2. Brück W, Pförtner R, Pham T, Zhang J, Hayardeny L, Piryatinsky V, Hanisch UK, Regen T et al (2012) Reduced astrocytic NF-κB activation by laquinimod protects from cuprizone-induced demyelination. Acta Neuropathol 124(3):411–24. doi:10.1007/s00401-012-1009-1
3. Skripuletz T, Bussmann JH, Gudi V, Koutsoudaki PN, Pul R, Moharregh-Khiabani D, Lindner M, Stangel M (2010) Cerebellar cortical demyelination in the murine cuprizone model. Brain Pathol 20(2):301–12. doi:10.1111/j.1750-3639.2009.00271.x
4. Kipp M, Clarner T, Dang J, Copray S, Beyer C (2009) The cuprizone animal model: new insights into an old story. Acta neuropathol 118(6):723–36. doi:10.1007/s00401-009-0591-3
5. Matsushima GK, Morell P (2001) The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol 11(1):107–16. doi:10.1111/j.1750-3639.2001.tb00385.x
6. Kondo A, Nakano T, Suzuki K (1987) Blood-brain barrier permeability to horseradish peroxidase in twitcher and cuprizone-intoxicated mice. Brain Res 425(1):186–90. doi:10.1016/0006-8993(87)90499-9
7. Brzezinski A (1997) Melatonin in humans. N Engl J Med 336(3):186–95. doi:10.1056/NEJM199701163360306
8. Carrillo-Vico A, Lardone PJ, Álvarez-Sánchez N, Rodríguez-Rodríguez A, Guerrero JM (2013) Melatonin: buffering the immune system. International journal of molecular sciences. Int J Mol Sci 14(4):8638–83. doi:10.3390/ijms14048638
9. Villapol S, Fau S, Renolleau S, Biran V, Charriaut-Marlangue C, Baud O (2011) Melatonin promotes myelination by decreasing white matter inflammation after neonatal stroke. Pediatr Res 69(1):51–5. doi:10.1203/PDR.0b013e3181fcb40b
10. Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J (2010) International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. Pharmacol Rev 62(3):343–80. doi:10.1124/pr.110.002832
11. Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S, Melan MA (2003) Melatonin receptors and their regulation: biochemical and structural mechanisms. Life Sci 72(20):2183–98. doi:10.1016/S0024-3205(03)00098-5
12. Molinari EJ, North PC, Dubocovich ML (1996) 2-[125I]iodo-5-methoxycarbonylamino-N-acetyltryptamine: a selective radioligand for the characterization of melatonin ML2 binding sites. Eur J Pharmacol 301(1):159–68. doi:10.1016/0014-2999(95)00870-5
13. Wang X (2009) The antiapoptotic activity of melatonin in neurodegenerative diseases. CNS Neurosci Ther 15(4):345–57. doi:10.1111/j.1755-5949.2009.00105.x
14. Esposito E, Cuzzocrea S (2010) Antiinflammatory activity of melatonin in central nervous system. Curr Neuropharmacol 8(3):228. doi:10.2174/157015910792246155
15. Esposito E, Paterniti I, Mazzon E, Bramanti P, Cuzzocrea S (2010) Melatonin reduces hyperalgesia associated with inflammation. J Pineal Res 49(4):321–31. doi:10.1111/j.1600-079X.2010.00796.x
16. Kang JC, Ahn M, Kim YS, Moon C, Lee Y, Wie MB, Lee YJ, Shin T (2001) Melatonin ameliorates autoimmune encephalomyelitis through suppression of intercellular adhesion molecule-1. J Vet Sci 2(2):85–9
17. Bahamonde C, Conde C, Agüera E, Lillo R, Luque E, Gascón F, Feijóo M, Cruz AH et al (2014) Elevated melatonin levels in natalizumab-treated female patients with relapsing-remitting multiple sclerosis: relationship to oxidative stress. Eur J Pharmacol 730:26–30. doi:10.1016/j.ejphar.2014.02.020
18. Azami K, Etminani M, Tabrizian K, Salar F, Belaran M, Hosseini A, Hosseini-Sharifabad A, Sharifzadeh M (2010) The quantitative evaluation of cholinergic markers in spatial memory improvement induced by nicotine–bucladesine combination in rats. Eur J Pharmacol 636(1):102–7. doi:10.1016/j.ejphar.2010.03.041
19. Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463(1):3–33. doi:10.1016/S0014-2999(03)01272-X
20. Vakilzadeh G, Khodagholi F, Ghadiri T, Darvishi M, Ghaemi A, Noorbakhsh F, Gorji A, Sharifzadeh M (2015) Protective effect of a cAMP Analogue on behavioral deficits and neuropathological changes in cuprizone model of demyelination. Mol Neurobiol 52(1):130–41. doi:10.1007/s12035-014-8857-8
21. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1):248–54
22. Ghadiri T, Sharifzadeh M, Khodagholi F, Modarres Mousavi SM, Hassanzadeh G, Zarrindast MR, Gorji A (2014) A novel traumatic brain injury model for induction of mild brain injury in rats. J Neurosci Methods 233:18–27. doi:10.1016/j.jneumeth
23. Kim JH, Budde MD, Liang H-F, Klein RS, Russell JH, Cross AH et al (2006) Detecting axon damage in spinal cord from a mouse model of multiple sclerosis. Neurobiol Dis 21(3):626–32. doi:10.1016/j.nbd.2005.09.009
24. Ghaemi A, Sajadian A, Khodaie B, Lotfinia AA, Lotfinia M, Aghabarari A, Khaleghi Ghadiri M, Meuth S et al (2014)

Immunomodulatory effect of toll-like receptor-3 ligand Poly I:C on cortical spreading depression. Mol Neurobiol. doi:10.1007/s12035-014-8995-z, **Nov 23**

25. Jafarian M, Karimzadeh F, Alipour F, Attari F, Lotfinia AA, Speckmann EJ, Zarrindast MR, Gorji A (2015) Cognitive impairments and neuronal injury in different brain regions of a genetic rat model of absence epilepsy. Neuroscience 298:161–70. doi:10.1016/j.neuroscience.2015.04.033
26. Liebetanz D, Merkler D (2006) Effects of commissural de-and remyelination on motor skill behaviour in the cuprizone mouse model of multiple sclerosis. Exp Neurol 202(1):217–24. doi:10.1016/j.expneurol.2006.05.032
27. Esposito E, Genovese T, Caminiti R, Bramanti P, Meli R, Cuzzocrea S (2009) Melatonin reduces stress-activated/mitogen-activated protein kinases in spinal cord injury. J Pineal Res 46(1):79–86. doi:10.1111/j.1600-079X.2008.00633.x
28. Srinivasan V, Lauterbach EC, Ho KY, Acuña-Castroviejo D, Zakaria R, Brzezinski A (2012) Melatonin in antinociception: its therapeutic applications. Curr Neuropharmacol 10(2):167. doi:10.2174/157015912800604489
29. Laste G, de Macedo IC, Rozisky JR, da Silva FR, Caumo W, Torres IL (2012) Melatonin administration reduces inflammatory pain in rats. J Pain Res 5:359–62. doi:10.2147/JPR.S34019
30. Hodges PW, Smeets RJ (2015) Interaction between pain, movement, and physical activity: short-term benefits, long-term consequences, and targets for treatment. Clin J Pain 31(2):97–107. doi:10.1097/AJP.0000000000000098
31. Curros-Criado MM, Herrero JF (2007) The antinociceptive effect of systemic gabapentin is related to the type of sensitization-induced hyperalgesia. J Neuroinflammation 4:15. doi:10.1186/1742-2094-4-15
32. Wang X, Fueroa BE, Stavrovskaya IG, Zhang Y, Sirianni AC, Zhu S et al (2009) Methazolamide and melatonin inhibit mitochondrial cytochrome C release and are neuroprotective in experimental models of ischemic injury. Stroke 40(5):1877–85. doi:10.1161/STROKEAHA.108.540765
33. Sun FY, Lin X, Mao LZ, Ge WH, Zhang LM, Huang YL et al (2002) Neuroprotection by melatonin against ischemic neuronal injury associated with modulation of DNA damage and repair in the rat following a transient cerebral ischemia. J Ppineal Res 33(1):48–56. doi:10.1034/j.1600-079X.2002.01891.x
34. Fuchs Y, Steller H (2011) Programmed cell death in animal development and disease. Cell 147(4):742–58. doi:10.1016/j.cell.2011.10.033
35. Negron JF, Lockshin RA (2004) Activation of apoptosis and caspase-3 in zebrafish early gastrulae. Dev Dyn 231(1):161–70. doi:10.1002/dvdy.20124
36. Andrabi SA, Sayeed I, Siemen D, Wolf G, Horn TF (2004) Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. FASEB J 18(7):869–71. doi:10.1096/fj.03-1031fje
37. Jang MH, Jung SB, Lee MH, Kim CJ, Oh YT, Kang I, Kim J, Kim EH (2005) Melatonin attenuates amyloid beta25-35-induced apoptosis in mouse microglial BV2 cells. Neurosci Lett 380(1):26–31. doi:10.1016/j.neulet.2005.01.003
38. Ling X, Zhang LM, Lu SD, Li XJ, Sun FY (1999) Protective effect of melatonin on injuried cerebral neurons is associated with bcl-2 protein over-expression. Zhongguo Yao Li Xue Bao 20(5):409–14
39. Hesse A, Wagner M, Held J, Brück W, Salinas-Riester G, Hao Z, Waisman A, Kuhlmann T (2010) In toxic demyelination oligodendroglial cell death occurs early and is FAS independent. Neurobio Dis 37(2):362–9. doi:10.1016/j.nbd.2009.10.016
40. Reiter RJ, Tan DX, Manchester LC, Qi W (2001) Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. Cell Biochem Biophys 34(2):237–56. doi:10.1385/CBB:34:2:237
41. Raasch J, Zeller N, Van Loo G, Merkler D, Mildner A, Erny D, et al. (2011) IκB kinase 2 determines oligodendrocyte loss by non-cell-autonomous activation of NF-κB in the central nervous system. Brain. awq359. doi: 10.1093/brain/awq359
42. Brambilla R, Hurtado A, Persaud T, Esham K, Pearse DD, Oudega M, Bethea JR (2009) Transgenic inhibition of astroglial NF-κB leads to increased axonal sparing and sprouting following spinal cord injury. J Neurochem 110(2):765–78. doi:10.1111/j.1471-4159.2009.06190.x
43. Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ (2005) A review of the multiple actions of melatonin on the immune system. Endocrine 27(2):189–200. doi:10.1385/ENDO:27:2:189
44. Epstein FH, Barnes PJ, Karin M (1997) Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336(15):1066–71. doi:10.1056/NEJM199704103361506
45. Garcia-Maurino S, Gonzalez-Haba MG, Calvo JR, Rafii-El-Idrissi M, Sanchez-Margalet V, Goberna R et al (1997) Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. J Immunol 159(2):574–81
46. Cristofanon S, Uguccioni F, Cerella C, Radogna F, Dicato M, Ghibelli L, Diederich M (2009) Intracellular prooxidant activity of melatonin induces a survival pathway involving NF-κB activation. Ann N Y Acad Sci 1171(1):472–8. doi:10.1111/j.1749-6632.2009.04896.x
47. Radogna F, Diederich M, Ghibelli L (2010) Melatonin: a pleiotropic molecule regulating inflammation. Biochem Pharmacol 80(12):1844–52. doi:10.1016/j.bcp.2010.07.041
48. Markus RP, Cecon E, Pires-Lapa MA (2013) Immune-pineal axis: nuclear factor κB (NF-kB) mediates the shift in the melatonin source from pinealocytes to immune competent cells. Int J Mol Sci 14(6):10979–97. doi:10.3390/ijms140610979
49. Willis D, Moore A, Frederick R, Willoughby D (1996) Heme oxygenase: a novel target for the modulation of inflammatory response. Nat Med 2(1):87–93. doi:10.1038/nm0196-87
50. Ganesh Yerra V, Negi G, Sharma SS, Kumar A (2013) Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy. Redox Biol 1(1):394–7. doi:10.1016/j.redox.2013.07.005
51. Tsunoda I, Fujinami RS (2002) Inside-Out versus Outside-In models for virus induced demyelination: axonal damage triggering demyelination. Springer Semin Immunopathol 24(2):105–25. doi:10.1007/s00281-002-0105-z
52. Deb C, Lafrance-Corey RG, Zoecklein L, Papke L, Rodriguez M, Howe CL (2009) Demyelinated axons and motor function are protected by genetic deletion of perforin in a mouse model of multiple sclerosis. J Neuropathol Exp Neurol 68(9):1037–48. doi:10.1097/NEN.0b013e3181b5417e
53. Bjartmar C, Trapp BD (2003) Axonal degeneration and progressive neurologic disability in multiple sclerosis. Neurotox Res 5(1-2):157–64
54. Kashani IR, Rajabi Z, Akbari M, Hassanzadeh G, Mohseni A, Eramsadati MK, Rafiee K, Beyer C et al (2014) Protective effects of melatonin against mitochondrial injury in a mouse model of multiple sclerosis. Exp Brain Res 232(9):2835–46. doi:10.1007/s00221-014-3946-5
55. Adamczyk-Sowa M, Pierzchala K, Sowa P, Polaniak R, Kukla M, Hartel M (2014) Influence of melatonin supplementation on serum antioxidative properties and impact of the quality of life in multiple sclerosis patients. J Physiol Pharmacol 65(4):543–50