Am J Neurodegener Dis 2021;10(5):57-68
www.AJND.us /ISSN:2165-591X/AJND0136370

Original Article

Medroxyprogesterone acetate attenuates demyelination, modulating microglia activation, in a cuprizone neurotoxic demyelinating mouse model

Maryam Mohammadi[1], Mahdad Abdi[1], Mehdi Alidadi[1], Wael Mohamed[2,3], Kazem Zibara[4], Iraj Ragerdi Kashani[1]

[1]Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran; [2]Department of Basic Medical Science, Kulliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia; [3]Clinical Pharmacology Department, Menoufia Medical School, Menoufia, Egypt; [4]PRASE and Biology Department, Faculty of Sciences, Lebanese University, Beirut, Lebanon

Received June 15, 2021; Accepted October 15, 2021; Epub October 15, 2021; Published October 30, 2021

Abstract: Clinical data reported a reduction of Multiple sclerosis (MS) symptoms during pregnancy when progesterone levels are high. Medroxyprogesterone acetate (MPA) is a synthetic progestin contraceptive with unknown neuroprotective effects. This study investigated the effect of a contraceptive dose of MPA on microglia polarization and neuroinflammation in the neurotoxic cuprizone (CPZ)-induced demyelinating mouse model of MS. Mice received 1 mg of MPA weekly, achieving similar serum concentrations in human contraceptive users. Results revealed that MPA therapy significantly reduced the demyelination in the corpus callosum. In addition, MPA treatment induced a significant reduction in microglia M1-markers (iNOS, IL-1β and TNF-α) while M2-markers (Arg-1, IL-10 and TGF-β) were significantly increased. Moreover, MPA resulted in a significant decrease in the number of iNOS positive cells (M1), whereas TREM-2 positive cells (M2) significantly increased. Furthermore, MPA decreased the protein expression levels of NF-κB and NLRP3 inflammasome as well as mRNA expression levels of the downstream product IL-18. In summary, MPA reduces the level of demyelination and has an anti-inflammatory role in CNS demyelination by inducing M2 microglia polarization and suppressing the M1 phenotype through the inhibition of NF-κB and NLRP3 inflammasome. Our results suggest that MPA should be a suitable contraceptive pharmacological agent in demyelinating diseases.

Keywords: Medroxyprogesterone acetate, multiple sclerosis, cuprizone, microglia, neuroinflammation, demyelination

Introduction

Multiple sclerosis (MS) is a neurodegenerative disorder characterized by myelin destruction, oligodendroglial loss, inflammation, axonal degeneration, astrogliosis and microgliosis [1, 2]. MS is more prevalent among female gender which suggests a possible role for hormonal and genetic factors [3, 4]. An early and common feature of the majority of MS pathologies is the activation of microglia in the damaged CNS [5].

Microglia, the resident macrophage cells of the central nervous system (CNS), are considered as immune guards capable of orchestrating a potent inflammatory response [6]. Traditionally, microglia were shown to be polarized into the pro-inflammatory phenotype (M1) or an anti-inflammatory (M2) phenotype [7]. M1 microglia are capable of producing and releasing reactive oxygen species (ROS), nitrogen reactive species (NRS), interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) [8, 9]. On the other hand, M2 microglia generates trophic factors such as brain derived neurotrophic factor (BDNF) and tumor growth factor-β (TGF-β) [6, 10].

Mechanisms of M1/M2 polarization associated with a broad spectrum of neurodegenerative diseases have been widely investigated [11]. Sex steroids were shown to have protective effects during the onset and progression of MS since clinical proves reported a dramatic reduc-

tion of MS symptoms during pregnancy, particularly when estrogen and progesterone levels are relatively high [12]. It has been reported that progesterone treatment attenuates M1-microglia polarization and enhances remyelination in animal models of MS [13, 14]. Hormonal contraceptives are an exogenous source of sex steroid hormones and could have a role in the development of MS [15, 16]. An epidemiologic study of contraceptive use and MS risk reported that contraceptive hormones could have different effects on the risk of autoimmunity, however this risk varied according to progestin content [17].

The identification of progesterone as a neuroprotective agent opens new perspectives for the use of progestogen-based drugs in MS. For instance, the most widely used progestin in progestin-only contraceptives is Medroxyprogesterone Acetate (MPA), a synthetic progestin derived from 17α-hydroxy-progesterone [18]. While the polarization of macrophages has been shown to be regulated by MPA [19]; nevertheless, the function of MPA in CNS microglia polarization is still unclear.

The MPA dose resulting into serum MPA concentrations in C57BL/6 mice comparable to those observed in women contraceptive users has been recently determined [20]. The purpose of the present study was to apply this MPA dosage in order to study its effect on demyelination, inflammatory cytokines, and microglial activation in the cuprizone (CPZ) mouse model of MS.

Material and methods

Animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) and Ethics Committees of Tehran University of Medical Science (TUMS), Tehran, Iran, (IR.TUMS.MEDICINE.REC.1398.838). Male C57BL/6 mice (6-8 weeks old, 20-25 g) were purchased from Pasteur institute (Karaj, Iran). Mice were maintained and housed at the Animal Breeding Center under pathogen-free conditions with constant temperature and a 12/12 hours' light/dark cycles. Surgical procedures were carried out under deep anesthesia.

Pharmacologic agent

Kleynhans and colleagues demonstrated that C57BL/6 mice treated by MPA with 1 mg/mouse/week could achieve serum concentrations similar to those observed in human contraceptive users [20]. In this study, MPA (150 mg, Iran hormone Co., Tehran, Iran) was diluted to 20 mg/ml in sterile phosphate-buffered saline (PBS; Sigma-Aldrich, USA). Mice were injected with 1 mg of MPA in 50 μl vehicle intramuscularly, into the right thigh muscle, once a week.

Induction of cuprizone and MPA treatment

A total of 30 male C57BL/6 mice were divided into three different groups with ten animals per group. Mice in the control (Ctrl) group were given standard normal diet for 6 weeks. The cuprizone (CPZ) group was given the same diet supplemented with 0.2% cuprizone (Sigma-Aldrich, USA) for 6 weeks. The medroxyprogesterone acetate group (CPZ+MPA) consisted of cuprizone-treated mice which also received an MPA injection every week, starting from the first week until the end of the experimental procedure.

Sacrifice and tissue collection

After 6 weeks of cuprizone administration, mice were deeply anesthetized with Ketamine (Sigma, St. Louis, USA)/Xylazine (Heidelberg, Germany), as follows. A stock solution was prepared in a syringe including 160 μL Ketamine 100 mg/mL (Sigma, St. Louis, USA), 160 μL Xylazine 2% (Heidelberg, Germany), and 1680 μL physiologic saline solution. A dosage of 80 mg/kg ketamine and 16 mg/kg xylazine (5/1 ratio), warmed to body temperature, was administered intraperitoneally at a volume of 10 μL/g body weight using 27 ¾ Gauge needles. Mice were then perfused with a transcardiac injection of PBS (Sigma-Aldrich, USA) and then fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, USA) [21]. Briefly, following complete anesthesia (animals were checked for toe-pinch reflex to check for pain reflex before any procedures were done), the animal was cut open below the diaphragm and the rib cage was cut rostrally on the lateral edges to expose the heart. A small hole was cut in the left ventricle and the needle was inserted into the aorta and clamped, then the right atrium was cut to allow flow. The animal was transcardially perfused with PBS wash for 4-5 minutes or until liver is cleared of blood. Next, the animal was transcardially perfused with 4% PFA for 4 minutes. The head was

removed (the animal was decapitated at a level even with forelimbs to ensure that the entire brainstem is included), skin trimmed off and the brain was post fixed in a small jar of 4% PFA for 24 hours. Brains were removed taking good attention to the corpus callosum above the fornix. Coronal brain sections were immersed in 4% PFA for 24 h then the tissue was dehydrated and embedded into paraffin blocks (Merck, Germany). Sections (5 μm) of the fixed tissues were prepared from the corpus callosum, deparaffinized and hydrated through a routine xylol and alcohol protocol. In order to analyze gene expression, mice were transcardially perfused with ice-cold Dulbecco's PBS (D-PBS, Sigma, USA). Thereafter, whole brains were rapidly snap frozen in liquid nitrogen and stored at -80°C until used.

Luxol fast blue staining

The presence of demyelination in the corpus callosum of cuprizone-treated mice was measured using Luxol Fast Blue (LFB, Gibco, USA) staining of formalin-fixed sections according to the protocol described by Acs et al. [22]. After deparaffinization and rehydration using decreasing grades of ethanol, coronal brain sections were incubated overnight in LFB solution (0.01) at 56°C and washed in ethanol and distilled water to remove the excess blue stain. In order to distinguish white matter from gray matter, the staining color was differentiated in a lithium carbonate solution (Merck, Germany) for 15 s. Slides were further passed through fresh xylene (Merck, Germany) for two times and mounted with Entellan (Merck, Germany). The sections were scanned and captured using an Olympus light microscope (Olympus CX310, Japan) equipped with a digital camera (Olympus, Japan). The quantification of LFB images was performed as previously described [23]. Briefly, the areas of demyelination (clear-white) to normal tissue (blue) were quantified using 10 random sections from each mouse using ImageJ software (Image J software, USA). Demyelination in each section was confirmed by monitoring adjacent sections. The percentage of demyelinated area was calculated by dividing the lesion size to the total area for each section.

Immunohistochemistry

Following deparaffinization and hydration, sections were incubated in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20), quenched in H_2O_2, and blocked at room temperature. Non-specific labeling was blocked using 0.1% bovine serum albumin (BSA, Sigma, USA) in 0.1% Triton X-100/PBS for 60 min. Sections were incubated overnight at 4°C with primary antibodies for -iNOS (1:500, Abcam, UK), TREM-2 (1:600, Antibodies-Online), Iba-1 (1:4000, Abcam, UK), NF-κB (1:500, SantaCruz Biotechnology, USA) and NLRP3 (1:400, Bioss, USA). After washing, the sections were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (Vectastain® ABC kit, Vector Laboratories) for 1 h at room temperature. Diaminobenzidine solution (DAB; DAKO, Germany) was added for 10 min, then slides were observed under a light microscope (Olympus IX-71; 40× objective) equipped with a Canon EOS digital camera. A total of four slices were analyzed for each animal (n=3 per group), with a distance of 150 μm in between. Quantification of Iba-1 was performed by measuring the immunostained areas with ImageJ (National Institutes of Health, Bethesda) and was demonstrated as the percentage of positive stained area [24]. For quantification of iNOS, TREM-2, NF-κB and NLRP3, the number of positive cells was measured.

Real time-PCR

Total RNA was isolated from brain tissues using QIAGEN RNeasy Kit (Qiagen, Tokyo, Japan), and then cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Quantitative real-time PCR (qRT-PCR) was performed by the StepOne RealTime PCR system (Applied Biosystems, USA). Cycling conditions for all analyses consisted of initial denaturation at 95°C during 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. Real-time PCR was carried out with RealQ Plus 2× Master Mix Green (Ampliqon, Denmark). Quantifcation was performed using 2-ΔΔCt; n=3 per group. The primer sequences are listed in **Table 1**. The specificity of PCR products was confirmed by melting curve analysis (data not shown). The expression levels of mRNAs were then reported relative to the β-actin reference gene.

Statistical analysis

Results are presented as mean values ± standard deviation of the mean (mean ± SD) and analyzed using Graph Pad Prism (Version 6.01, CA, and the USA). The Kolmogorov-Smirnov test

Table 1. List of primers used for real time-PCR

	Forward primer	Reverse primer
IL-10	GCCCAGAAATCAAGGAGCATT	GCTCCACTGCCTTGCTTTTA
IL-1β	TGCCACCTTTTGACAGTGATG	GGTCCACGGGAAAGACAC
TNF-α	GAACTGGCAGAAGAGGCACT	TTGAGAAGATGATCTGAGTGTGG
TGF-β	CAAGGGCTACCATGCCAACT	GTACTGTGTGTCCAGGCTCCAA
IL-18	GCCTGATATCGACCGAACA	CCTTCCATCCTTCACAGATAGG
Arge1	TCACCTGAGCTTTGATGTCG	CTGAAAGGAGCCCTGTCTTG
iNOS	CAAGCACCTTGGAAGAGGAG	AAGGCCAAACACAGCATACC
β-actin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC

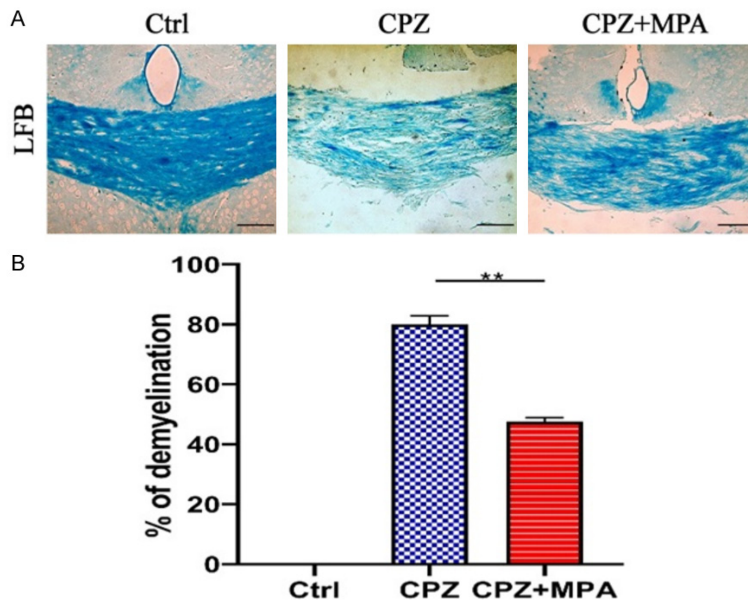

Figure 1. The effect of MPA treatment on cuprizone induced demyelination. A. Representative images of LFB-stained sections in the corpus callosum of control (Ctrl), cuprizone alone (CPZ) or combined with MPA (CPZ+MPA). Results showed that MPA accelerated demyelination in the corpus callosum of CPZ-induced mice. B. Quantitative analysis for LFB in the corpus callosum. Scale bar =100 μm. Data are presented as the mean ± SD. n=3 per group. Significance is indicated by $^{**}P<0.01$.

was used for normality evaluation. Comparison between the studied groups were performed by a parametric one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Results of PCR analysis from five independent experiments were normalized against β-actin and are presented as the percentage of control. Significance is indicated by $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Results

MPA therapy reduced the severity of demyelination in CPZ-treated mice

The MPA dose of 1 mg/mouse/week was used in this study since it was previously demonstrated to achieve MPA serum concentrations in mice similar to those observed in human contraceptive users [20]. The effect of this MPA dose was then used to investigate the myelin content and the extent of demyelination area in the corpus callosum by performing luxol fast blue (LFB) staining. Administration of cuprizone (CPZ) diet for 6 weeks caused a robust demyelination in the corpus callosum, compared to control (Ctrl) mice (**Figure 1A**). However, treatment by MPA therapy (CPZ+MPA) dramatically decreased this CPZ-induced demyelination (**Figure 1A**). Quantification of LFB images measuring the amount of myelin loss for each brain was also performed. Results confirmed that CPZ caused a significant demyelination in the corpus callosum, in comparison to controls (**Figure 1B**). Moreover, the percentage of demyelination was significantly ($^{**}P<0.01$) reduced in MPA treated mice (CPZ+MPA), compared to the CPZ group (48% vs 80%, respectively) (**Figure 1B**).

MPA modulates microglia polarization from M1 to M2 phenotype at the transcriptional level

Microglia polarization is induced over time and represents the switching of microglia from an activated M2- towards a classical M1-phenotype. In this study, we investigated whether MPA treatment, administered once a week for the six weeks' period of CPZ demyelination, can induce switching between M1 and M2 phenotypes. The polarization of microglia was first analyzed at the transcriptional level by quantitative real-time PCR, in the corpus callosum of mice at week 6. The expression profiling was performed using M1- (iNOS, TNF-α, IL-1β) (**Figure 2A**) and M2-assosiated (Arg-1, TGF-β, IL-10) markers (**Figure 2B**); respectively. Results revealed that the CPZ group of mice demonstrated significantly higher mRNA expression

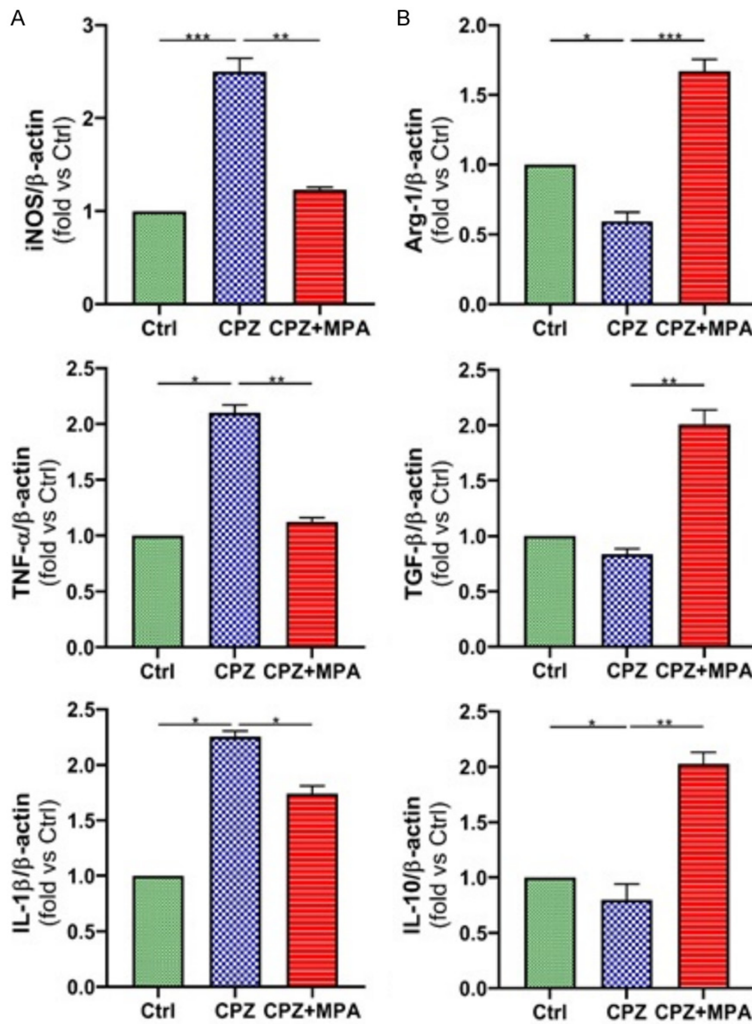

Figure 2. The effect of MPA on the expression levels of microglia associated genes. A. Quantitative real time PCR for mRNA expression levels of M1-microglia associated markers iNOS, TNFα and IL-1β. The levels of these mRNAs were upregulated in CPZ mice; however, they were significantly decreased following MPA administration. B. Quantitative real time PCR for mRNA expression levels of M2-microglia associated markers Arg-1, TGF-β and IL-10. Results showed a significant decrease of these markers in CPZ mice, which was inhibited by MPA therapy. Data are presented as the mean ± SD. n=3 per group. Significance is indicated by $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

levels of M1-microglia markers (iNOS, TNF-α and IL-1β), in comparison to controls (**Figure 2A**). However, MPA treatment (CPZ+MPA) resulted in a significant decrease in the expression of M1 markers (iNOS, TNF-α and IL-1β), in comparison to CPZ mice (**Figure 2A**). This result highlights the anti-inflammatory role of MPA in CNS demyelination by suppressing M1-microglia polarization. In contrast, the expression of M2-related markers (Arg-1, TGF-β and IL-10) was significantly ($^{*}P<0.05$) decreased in CPZ mice, in comparison to controls. However, the transcriptional expression levels of M2 markers (Arg-1, TGF-β and IL-10) were significantly increased following MPA therapy (CPZ+MPA), in comparison to the CPZ group (**Figure 2B**). These results clearly indicate the anti-inflammatory role of MPA therapy in CNS demyelination by inducing M2 microglia polarization and suppressing the M1 phenotype.

MPA modulates microglia activation and polarization at the translational level

Microglia was then investigated at the protein level in the corpus callosum using immunohistochemistry (IHC) staining. Microglia activation was examined using Iba-1 while microglia conversion was explored using iNOS and TREM-2, representing the M1 and M2 phenotypes; respectively (**Figure 3A**). Consistent with the transcriptional data, quantification of IHC images demonstrated that induction of demyelination by CPZ caused a significant ($^{**}P<0.001$) increase in the number of Iba-1 and iNOS positive cells in CPZ mice, in comparison to controls (**Figure 3B** and **3C**, respectively). However, MPA treatment (CPZ+MPA) resulted in a significant ($^{*}p<0.05$) decrease in the number of Iba-1 (**Figure 3B**) and iNOS (**Figure 3C**) positive cells, in comparison to CPZ mice. On the other hand, the expression of TREM-2 positive cells showed an opposite trend and decreased significantly ($^{*}P<0.05$) in CPZ mice, in comparison to controls (**Figure 3D**). Interestingly, MPA therapy also reversed the trend and increased significantly ($^{**}P<0.01$) the number of TREM-2 positive cells in comparison to the CPZ group (**Figure 3D**).

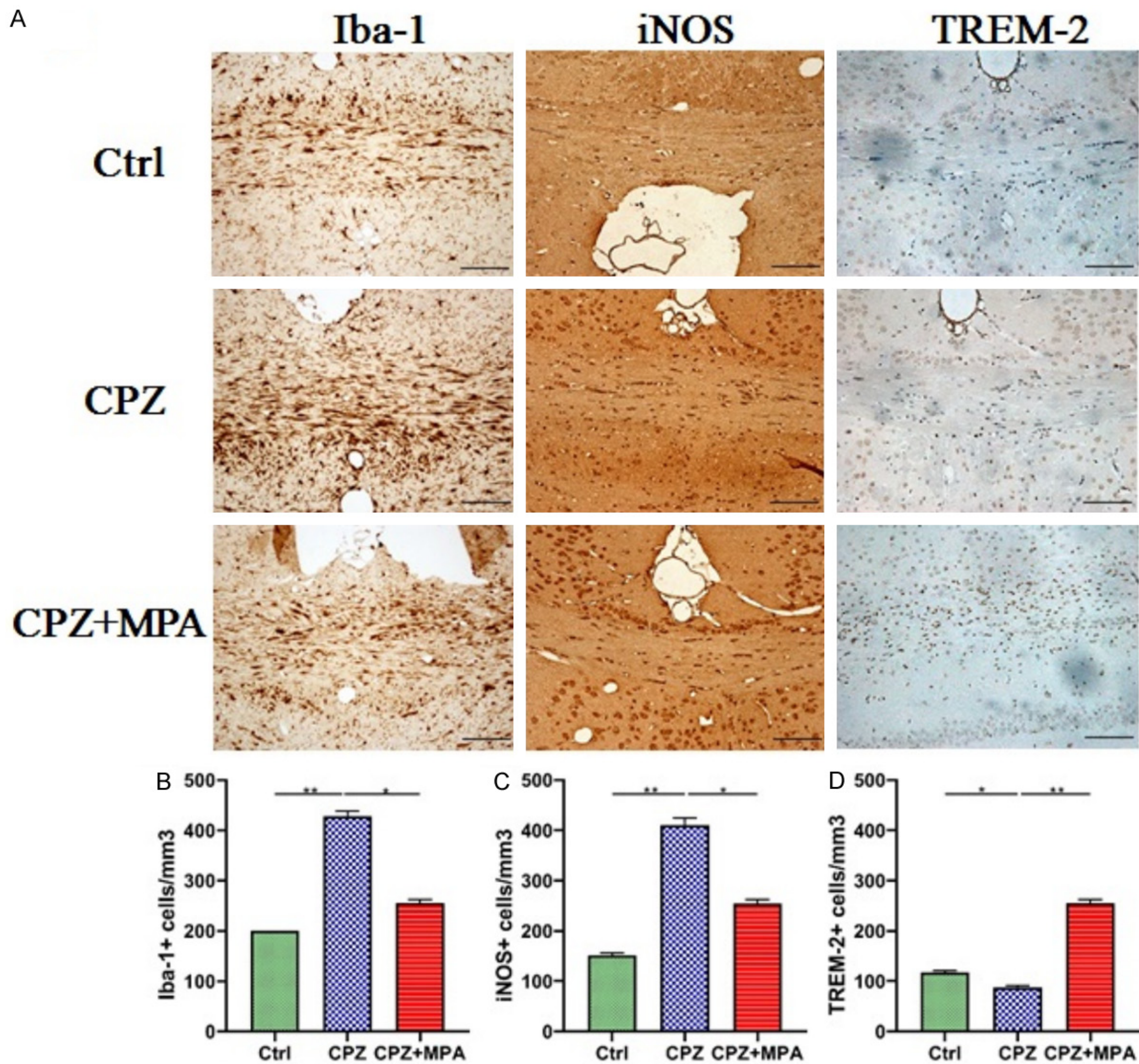

Figure 3. Immunohistochemistry (IHC) staining for markers of M1 and M2 microglia. A. Representative images of IHC staining for total microglia (Iba-1), M1-microglia phenotype (iNOS) and M2-microglia phenotype (TREM-2) in the corpus callosum. These are displayed for control (Ctrl), cuprizone alone (CPZ) or combined with MPA (CPZ+MPA) groups. B-D. Quantitative analysis of the number of Iba-1, iNOS and TREM-2 positive cells in the corpus callosum of different groups. Results showed a persistent upregulation of Iba-1 and iNOS positive cells and a significant downregulation of TREM-2 positive cells in microglia of CPZ mice, in comparison to the control group. However, administration of MPA caused an increase in TREM-2 positive cells while inhibiting Iba-1 and iNOS positive cells, in comparison to CPZ group. Scale bar =100 μm. Data are presented as the mean ± SD. n=3 per group. Significance is indicated by $^{*}P<0.05$ and $^{**}P<0.01$.

Taken together, these data clearly demonstrate a suppressing effect of MPA therapy on M1 microglia phenotype accompanied by a shift in microglia polarization toward M2-phenotype, both at the transcriptional and translation levels, promoting a hemostatic state.

NLRP3 inflammasome regulates MPA-mediated polarization of microglia

In order to investigate the mechanism by which MPA would suppress the M2 inflammatory microglia phenotype, we then determined the protein levels of NF-κB and NLRP3 inflammasome in the corpus callosum of mice, using immunohistochemistry (**Figure 4A**). Quantification of data demonstrated a prominent and significant ($^{**}P<0.01$) increase in the protein expression levels of NF-κB (**Figure 4B**) and NLRP3 (**Figure 4C**) in the CPZ demyelinated group, in comparison to controls. However, MPA therapy significantly ($^{*}P<0.05$) suppressed the protein levels of NF-κB and NLRP3, in comparison to CPZ mice (**Figure 4B** and **4C**, respectively).

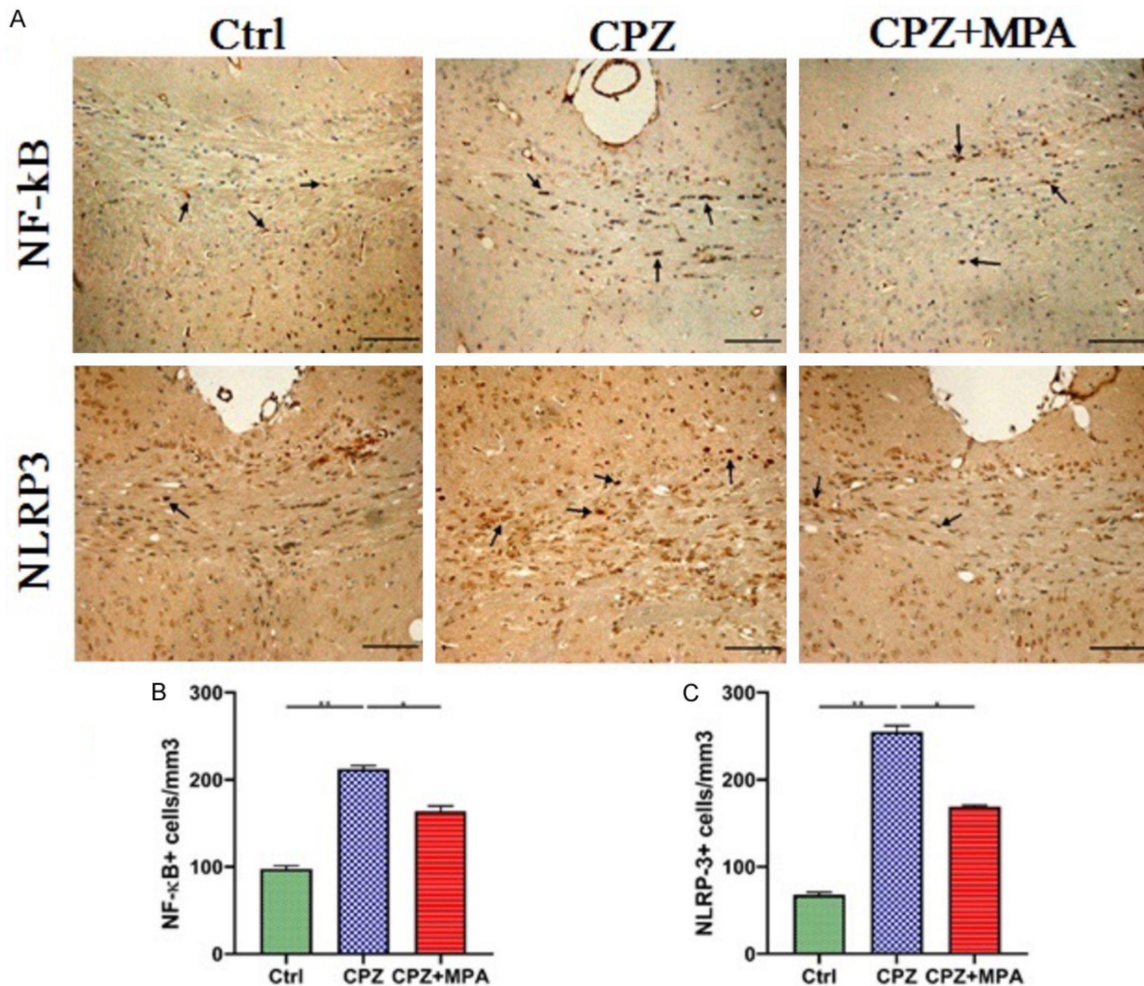

Figure 4. The effect of MPA on protein expression levels of NF-κB and NLRP3 inflammasome. A. Representative captures of IHC staining for NF-κB and NLRP3 proteins in the corpus callosum of control (Ctrl), cuprizone alone (CPZ) or combined with MPA (CPZ+MPA) groups. B, C. Quantitative analysis for NF-κB and NLRP3 protein expression levels. NF-κB and NLRP3 levels increased in CPZ mice, in comparison to controls. However, MPA administration significantly suppressed this increase, in comparison to CPZ mice. Scale bar =100 μm. Data are presented as the mean ± SD. n=3 per group. Significance is indicated by *P<0.05 and **P<0.01.

Since NLRP3 inflammasome plays a significant role in MS, the effects of MPA treatment on IL-18, the downstream end-product of NLRP3 was then analyzed by quantitative real time-PCR (**Figure 5**). mRNA expression levels of cleaved-IL-18 were shown to be significantly (***P<0.001) higher in CPZ group, compared to control mice (**Figure 5**). Interestingly, MPA administration caused a significant (**P<0.01) decrease in transcript levels of IL-18, in comparison to the CPZ group (**Figure 5**).

Taken together, these finding indicate that the mechanism by which MPA would induce M1 to M2 phenotype is achieved through the inhibition of NF-κB and NLRP3.

Discussion

Medroxyprogesterone acetate (MPA) is the most widely prescribed pill in progestin-only contraceptives [18]. In this study, we investigated the effects of a contraceptive dose of MPA on microglia polarization in CPZ mice. Evaluation of myelin indicated that MPA attenuated CPZ-induced demyelination in the corpus callosum. Subsequent histopathological analysis revealed that MPA administration decreased protein expression levels for M1 microglia phenotype markers and inhibited mRNA expression levels of pro-inflammatory cytokines. Previous studies examining the neuroprotective effects of MPA have reported conflicting

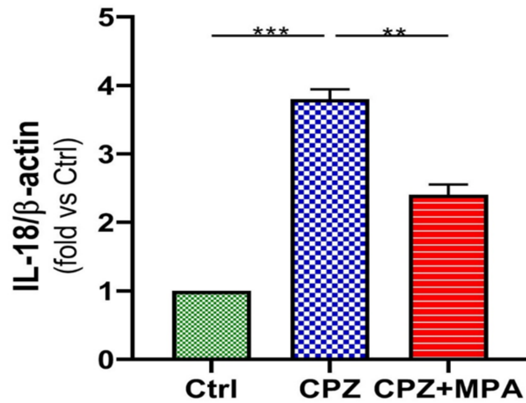

Figure 5. The effect of MPA on IL-18 transcript levels. Quantitative real-time PCR for IL-18 mRNA expression levels in the various groups. IL-18 transcript levels increased significantly in the CPZ group, in comparison to controls. However, MPA treatment suppressed this increase, in comparison to CPZ mice. Data are presented as the mean ± SD. n=3 per group. Significance is indicated by $^{**}P<0.01$ and $^{***}P<0.001$.

results. Some studies reported that MPA has no neuroprotective properties and failed to stimulate the formation of new myelin after a demyelinating insult with lysolecithin [25, 26]. However, other reports established that MPA could ameliorate inflammation; hence, it could be used as a therapeutic agent for neuroinflammatory and autoimmune disorders [27, 28]. It was also reported that MPA has neuroprotective effects and reduced microglial and macrophage activity in neuroinflammation disease [29]. At physiological levels, MPA was suggested to suppress innate and adaptive immune mechanisms [30, 31]. However, the above studies were not derived from animal models and concentrations used in most studies could not be translated to serum MPA levels present in contraceptive users.

In the present study, we evaluated the administration of serum concentration levels of MPA, similar to those observed among contraceptive users, on microglia conversion in a CPZ-induced demyelination mouse model of MS. It has been demonstrated that different states of M1/M2 microglia polarization can participate in the onset and progression of neurodegenerative diseases such as MS [32]. The present demyelination model reveled that mRNA expression levels of M1-specific microglia pro-inflammatory cytokines, such as IL-1β, TNF-α and iNOS [9, 33], were remarkably upregulated in mice receiving CPZ. However, MPA treatment significantly suppressed the expression of these cytokines. Our results were consistent with a previous report in which MPA treatment decreased the production of TNF-α pro-inflammatory cytokine, in the white matter after spinal cord injury [29]. Furthermore, our data showed that mRNA expression levels of microglia M2-specific anti-inflammatory cytokines, including IL-10, TGF-β and Arg1 [10, 34], were decreased in the corpus callosum of CPZ mice; however, they were significantly enhanced by MPA treatment. Moreover, analyses of iNOS and Arg1 levels allow the determination of the polarization state of microglia [14]. This study demonstrated, for the first time, that MPA-treated mice expressed a lower level of iNOS and a higher level of Arg-1, both of which are involved in L-arginine metabolism [35].

We established in this study that MPA can shift microglia toward an anti-inflammatory phenotype (M2) in demyelinated corpus callosum. Indeed, MPA caused an increase in the expression of IL-10, TGF-β and Arg1 and a decrease in the expression of IL-1β, TNF-α and iNOS. To validate these observations, we evaluated microglia activation by IHC staining, which confirmed that MPA treatment reduced the number of Iba-1 positive cells in CPZ-treated mice. In fact, phenotypic distinction between polarized microglia involves differential expression of cell surface receptors, among which TREM-2 are typically associated with M2 activation [36] whereas iNOS is considered as a hallmark of M1 microglia [37]. We found that CPZ treatment increased the protein expression levels of iNOS while reducing those of TREM2. In contrast, MPA administration significantly reduced the expression of iNOS while promoting those of TREM-2. Therefore, an increase in the expression of the M2 marker TREM2 and a reduction in the M1 marker iNOS, indicated a protective role of MPA treatment through modulating microglia status. In agreement, it has been reported that MPA is capable of switching the M1 macrophage phenotype into an M2 phenotype with downregulation of IL-1β and iNOS while upregulating IL-10 [19]. The latter study also suggested that these effects might be mediated through the glucocorticoid receptor that inhibit the function of NF-κB, a critical transcription factor for the expression of proinflammatory cytokines [19].

Inflammasome activation participate in neuroimmune response including secretion of inflammatory cytokines [38]. Inflammasomes are intracellular multi-protein complexes serving as major components of the immune system [39]. The NLRP3 inflammasome is present in many cells. In the CNS, a great number of studies have shown that the NLRP3 inflammasome is mainly localized in microglia [40-43]. A possible mechanism underlying the effect of MPA is its inhibition to NLRP3 inflammasome activation. In this study, we explored the level of NLRP3 inflammasome and IL-18 in the corpus callosum to elucidate whether the anti-inflammatory effect of MPA was associated with it. Here, we found that the NLRP3 inflammasome was activated while the pro-inflammatory cytokine IL-18 was released, following cuprizone treatment. In fact, we showed that MPA therapy decreased NLRP3 protein levels and IL-18 mRNA expression levels, in comparison to CPZ mice, which has not been reported previously. It is worth noting that several studies suggested that an increased expression of NLRP3 inflammasome, following CPZ treatment, could be considered as one of the mechanisms inducing microglia accumulation and promoting microglial M1 activation [14, 44]. On the other hand, the suppression in the expression of NLRP3 protein and IL-18 mRNA was paralleled with a reduction in M1 microglia activation and an enhancement in M2 phenotype. These findings suggest that the NLRP3 inflammasome may promote M1 microglia and increase the secretion of pro-inflammatory cytokines. Thus, the anti-inflammatory effect of MPA may be related to the inhibition of activation of both NLRP3 inflammasome and M1 microglia.

Furthermore, as a transcription factor, nuclear factor kappa B (NF-κB) also plays a key role in the activation and initiation of NLRP3 inflammasome in macrophages, since NF-κB-dependent signals positively regulate NLRP3 expression [45, 46]. In fact, NF-κB is a critical regulator of immune and inflammatory responses [47]. In mammalian cells, five NF-κB subunits have been identified, including P50, p52, p65 (Rel-A), c-Rel and Rel-B proteins [48]. In microglia, the NF-κB signaling is conducted through P65 (Re1A) subunit that translocates into the nucleus and initiates the transcription of proinflammatory cytokines [49]. In addition, activation of NF-κB was detected in the active lesions of microglia in MS patients, EAE model and in a CPZ model of MS [50]. Moreover, pro-inflammatory cytokines related to M1 phenotype, including IL-1β, TNF-α, iNOS, and NLRP3 inflammasome have been shown to be under tight regulation by NF-κB in microglia [51]. We evaluated whether NF-κB is involved in the inhibitory effect of MPA on NLRP3 inflammasome activation and microglia phenotype. In this study, we observed a prominent elevation in the protein levels of NF-κB P65, in accordance with a previous report demonstrating that NF-κB P65 mRNA expression level are increased after 5 weeks of cuprizone treatment [50]. On the other hand, MPA treatment decreased pro-inflammatory cytokines in CPZ induced demyelination by inhibiting the protein levels of NF-κB. In other words, along with the inhibition of NF-κB, the expression of NLRP3 inflammasome is decreased and microglia immunophenotype is regulated.

Concomitant with a downregulation in M1 phenotypic markers. The effect of CPZ on myelin distraction is related to the presence of TNF-α and iNOS cytokines secreted by microglia and inducing oligodendrocyte degeneration and subsequently demyelination [52, 53]. We evaluated demyelination by LFB staining [22] and observed that CPZ-induced demyelination was alleviated in MPA treated mice. Our data demonstrated that MPA ameliorates CPZ-induced demyelination through suppression of neuroinflammation.

The limitations of our study include the low number of samples that were available for histological and biochemical assays, thus decreasing the power of statistical tests to identify significant differences between groups. In addition, the effect of MPA hormone on cells involved in myelin repair (oligodendrocytes and oligodendrocyte progenitor cells) was not analyzed, therefore, we could not assesse the amount of remyelination. Although the above described theories serve as an initial hypothesis, it is clear that more research is needed to better understand the exact mechanism through which MPA may exert a protective effect on demyelination of the CNS. Moreover, future studies are needed to determine whether the effect of contemporary contraceptives in neurodegenerative disease could vary by progestin content.

Conclusion

Overall, we demonstrated, for the first time, that MPA alleviates CPZ-induced demyelination in vivo by blocking the activation of NLRP3 inflammasome and regulating the immunophenotype of microglia via inhibition of the NF-κB signaling pathway. Our results suggest that MPA should be a suitable contraceptive agent in demyelinating diseases.

Acknowledgements

The current study was supported by a grant (number 98-3-101-43373) from the Tehran University of Medical Sciences and Health Services, Tehran, Iran.

Disclosure of conflict of interest

None.

Address correspondence to: Iraj Ragerdi Kashani, Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Poursina Street, Tehran, Iran. Tel: +98-21-66419072; E-mail: ragerdi@tums.ac.ir; Kazem Zibara, PRASE and Biology Department, Faculty of Sciences, Lebanese University, Beirut, Lebanon. Tel: +961-70-939994; E-mail: kzibara@ul.edu.lb

References

[1] Ozsvár A, Szipőcs R, Ozsvár Z, Baka J, Barzó P, Tamás G and Molnár G. Quantitative analysis of lipid debris accumulation caused by cuprizone induced myelin degradation in different CNS areas. Brain Res Bull 2018; 137: 277-284.
[2] Stadelmann C, Wegner C and Brück W. Inflammation, demyelination, and degeneration-recent insights from MS pathology. Biochim Biophys Acta 2011; 1812: 275-282.
[3] Avila M, Bansal A, Culberson J and Peiris AN. The role of sex hormones in multiple sclerosis. Eur Neurol 2018; 80: 93-99.
[4] Harbo HF, Gold R and Tintoré M. Sex and gender issues in multiple sclerosis. Ther Adv Neurol Disord 2013; 6: 237-248.
[5] Goldmann T and Prinz M. Role of microglia in CNS autoimmunity. Clin Dev Immunol 2013; 2013: 208093.
[6] Bachiller S, Jiménez-Ferrer I, Paulus A, Yang Y, Swanberg M, Deierborg T and Boza-Serrano A. microglia in neurological diseases: a road map to brain-disease dependent-inflammatory response. Front Cell Neurosci 2018; 12: 488.
[7] Song GJ and Suk K. Pharmacological modulation of functional phenotypes of microglia in neurodegenerative diseases. Front Aging Neurosci 2017; 9: 139.
[8] Subramaniam SR and Federoff HJ. Targeting microglial activation states as a therapeutic avenue in Parkinson's disease. Front Aging Neurosci 2017; 9: 176.
[9] Tang Y and Le W. Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol Neurobiol 2016; 53: 1181-1194.
[10] Cherry JD, Olschowka JA and O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation 2014; 11: 98.
[11] Tang Y. Editorial: microglial polarization in the pathogenesis and therapeutics of neurodegenerative diseases. Front Aging Neurosci 2018; 10: 154.
[12] McCombe PA. The short and long-term effects of pregnancy on multiple sclerosis and experimental autoimmune encephalomyelitis. J Clin Med 2018; 7: 494.
[13] Ghoumari AM, Abi Ghanem C, Asbelaoui N, Schumacher M and Hussain R. Roles of progesterone, testosterone and their nuclear receptors in central nervous system myelination and remyelination. Int J Mol Sci 2020; 21: 3163.
[14] Aryanpour R, Pasbakhsh P, Zibara K, Namjoo Z, Beigi Boroujeni F, Shahbeigi S, Kashani IR, Beyer C and Zendehdel A. Progesterone therapy induces an M1 to M2 switch in microglia phenotype and suppresses NLRP3 inflammasome in a cuprizone-induced demyelination mouse model. Int Immunopharmacol 2017; 51: 131-139.
[15] Hernán MA, Hohol MJ, Olek MJ, Spiegelman D and Ascherio A. Oral contraceptives and the incidence of multiple sclerosis. Neurology 2000; 55: 848-854.
[16] Alonso A, Jick SS, Olek MJ, Ascherio A, Jick H and Hernán MA. Recent use of oral contraceptives and the risk of multiple sclerosis. Arch Neurol 2005; 62: 1362-1365.
[17] Hellwig K, Chen LH, Stancyzk FZ and Langer-Gould AM. Oral contraceptives and multiple sclerosis/clinically isolated syndrome susceptibility. PLoS One 2016; 11: e0149094.
[18] Giatti S, Melcangi RC and Pesaresi M. The other side of progestins: effects in the brain. J Mol Endocrinol 2016; 57: R109-126.
[19] Tsai YC, Tseng JT, Wang CY, Su MT, Huang JY and Kuo PL. Medroxyprogesterone acetate drives M2 macrophage differentiation toward a phenotype of decidual macrophage. Mol Cell Endocrinol 2017; 452: 74-83.
[20] Kleynhans L, Du Plessis N, Allie N, Jacobs M, Kidd M, van Helden PD, Walzl G and Ronacher

K. The contraceptive depot medroxyprogesterone acetate impairs mycobacterial control and inhibits cytokine secretion in mice infected with mycobacterium tuberculosis. Infect Immun 2013; 81: 1234-1244.
[21] Ghareghani M, Ghanbari A, Dokoohaki S, Farhadi N, Hosseini SM, Mohammadi R and Sadeghi H. Methylprednisolone improves lactate metabolism through reduction of elevated serum lactate in rat model of multiple sclerosis. Biomed Pharmacother 2016; 84: 1504-1509.
[22] Acs P, Kipp M, Norkute A, Johann S, Clarner T, Braun A, Berente Z, Komoly S and Beyer C. 17beta-estradiol and progesterone prevent cuprizone provoked demyelination of corpus callosum in male mice. Glia 2009; 57: 807-814.
[23] Noorzehi G, Pasbakhsh P, Borhani-Haghighi M, Kashani IR, Madadi S, Tahmasebi F, Nekoonam S and Azizi M. Microglia polarization by methylprednizolone acetate accelerates cuprizone induced demyelination. J Mol Histol 2018; 49: 471-479.
[24] Borhani-Haghighi M, Kashani IR, Mohamadi Y and Pasbakhsh P. Embryonic intraventricular transplantation of neural stem cells augments inflammation-induced prenatal brain injury. J Chem Neuroanat 2018; 94: 54-62.
[25] Hussain R, El-Etr M, Gaci O, Rakotomamonjy J, Macklin WB, Kumar N, Sitruk-Ware R, Schumacher M and Ghoumari AM. Progesterone and nestorone facilitate axon remyelination: a role for progesterone receptors. Endocrinology 2011; 152: 3820-3831.
[26] Schumacher M, Hussain R, Gago N, Oudinet JP, Mattern C and Ghoumari AM. Progesterone synthesis in the nervous system: implications for myelination and myelin repair. Front Neurosci 2012; 6: 10.
[27] Bamberger CM and Schulte HM. Molecular mechanisms of dissociative glucocorticoid activity. Eur J Clin Invest 2000; 30 Suppl 3: 6-9.
[28] Topsakal C, Kilic N, Erol FS, Kaplan M, Akdemir I, Tiftikci M and Gursu F. Medroxyprogesterone acetate, enoxaparin and pentoxyfylline cause alterations in lipid peroxidation, paraoxonase (PON1) activities and homocysteine levels in the acute oxidative stress in an experimental model of spinal cord injury. Acta Neurochir (Wien) 2002; 144: 1021-1031.
[29] Sahin B, Albayrak BS, Ismailoglu O and Gorgulu A. The effects of medroxy progesterone acetate on the pro-inflammatory cytokines, TNF-alpha and IL-1beta in the early phase of the spinal cord injury. Neurol Res 2011; 33: 63-67.
[30] Huijbregts RPH, Michel KG and Hel Z. Corrigendum to “effect of progestins on immunity: medroxyprogesterone but not norethisterone or levonorgestrel suppresses the function of T cells and pDCs” [contraception 90 (2014) 123-129]. Contraception 2016; 94: 578.
[31] Hel Z, Stringer E and Mestecky J. Sex steroid hormones, hormonal contraception, and the immunobiology of human immunodeficiency virus-1 infection. Endocr Rev 2010; 31: 79-97.
[32] Chu F, Shi M, Zheng C, Shen D, Zhu J, Zheng X and Cui L. The roles of macrophages and microglia in multiple sclerosis and experimental autoimmune encephalomyelitis. J Neuroimmunol 2018; 318: 1-7.
[33] Aguzzi A, Barres BA and Bennett ML. Microglia: scapegoat, saboteur, or something else? Science 2013; 339: 156-161.
[34] Saijo K and Glass CK. Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol 2011; 11: 775-787.
[35] Rath M, Müller I, Kropf P, Closs EI and Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. Front Immunol 2014; 5: 532.
[36] Cignarella F, Filipello F, Bollman B, Cantoni C, Locca A, Mikesell R, Manis M, Ibrahim A, Deng L, Benitez BA, Cruchaga C, Licastro D, Mihindukulasuriya K, Harari O, Buckland M, Holtzman DM, Rosenthal A, Schwabe T, Tassi I and Piccio L. TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis. Acta Neuropathol 2020; 140: 513-534.
[37] Lisi L, Ciotti GM, Braun D, Kalinin S, Currò D, Dello Russo C, Coli A, Mangiola A, Anile C, Feinstein DL and Navarra P. Expression of iNOS, CD163 and ARG-1 taken as M1 and M2 markers of microglial polarization in human glioblastoma and the surrounding normal parenchyma. Neurosci Lett 2017; 645: 106-112.
[38] Lang Y, Chu F, Zhang W, Zheng C, Zhu J, Cui L and Shen D. Role of inflammasomes in neuroimmune and neurodegenerative diseases: a systematic review. Mediators Inflamm 2018; 2018: 1549549.
[39] Strowig T, Henao-Mejia J, Elinav E and Flavell R. Inflammasomes in health and disease. Nature 2012; 481: 278-286.
[40] Gustin A, Kirchmeyer M, Koncina E, Felten P, Losciuto S, Heurtaux T, Tardivel A, Heuschling P and Dostert C. NLRP3 inflammasome is expressed and functional in mouse brain microglia but not in astrocytes. PLoS One 2015; 10: e0130624.
[41] Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Caneda C, Guarnieri P, Ruderisch N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA and Wu JQ. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci 2014; 34: 11929-11947.

[42] Hou B, Zhang Y, Liang P, He Y, Peng B, Liu W, Han S, Yin J and He X. Inhibition of the NLRP3-inflammasome prevents cognitive deficits in experimental autoimmune encephalomyelitis mice via the alteration of astrocyte phenotype. Cell Death Dis 2020; 11: 377.
[43] Fan Z, Liang Z, Yang H, Pan Y, Zheng Y and Wang X. Tenuigenin protects dopaminergic neurons from inflammation via suppressing NLRP3 inflammasome activation in microglia. J Neuroinflammation 2017; 14: 256.
[44] Jha S, Srivastava SY, Brickey WJ, Iocca H, Toews A, Morrison JP, Chen VS, Gris D, Matsushima GK and Ting JP. The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and interleukin-18. J Neurosci 2010; 30: 15811-15820.
[45] Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V and Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 2009; 183: 787-791.
[46] Zheng Y, Lilo S, Brodsky IE, Zhang Y, Medzhitov R, Marcu KB and Bliska JB. A Yersinia effector with enhanced inhibitory activity on the NF-κB pathway activates the NLRP3/ASC/caspase-1 inflammasome in macrophages. PLoS Pathog 2011; 7: e1002026.
[47] Gupta SC, Sundaram C, Reuter S and Aggarwal BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 2010; 1799: 775-787.
[48] Oeckinghaus A and Ghosh S. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol 2009; 1: a000034.
[49] Dresselhaus EC and Meffert MK. Cellular specificity of NF-κB function in the nervous system. Front Immunol 2019; 10: 1043.
[50] Ghaiad HR, Nooh MM, El-Sawalhi MM and Shaheen AA. Resveratrol promotes remyelination in cuprizone model of multiple sclerosis: biochemical and histological study. Mol Neurobiol 2017; 54: 3219-3229.
[51] Ganbold T, Bao Q, Zandan J, Hasi A and Baigude H. Modulation of microglia polarization through silencing of NF-κB p65 by functionalized curdlan nanoparticle-mediated RNAi. ACS Appl Mater Interfaces 2020; 12: 11363-11374.
[52] Praet J, Guglielmetti C, Berneman Z, Van der Linden A and Ponsaerts P. Cellular and molecular neuropathology of the cuprizone mouse model: clinical relevance for multiple sclerosis. Neurosci Biobehav Rev 2014; 47: 485-505.
[53] Raposo C, Nunes AK, Luna RL, Araújo SM, da Cruz-Höfling MA and Peixoto CA. Sildenafil (Viagra) protective effects on neuroinflammation: the role of iNOS/NO system in an inflammatory demyelination model. Mediators Inflamm 2013; 2013: 321460.