

Original Article

Crosstalk between BDNF and TNF α in brain versus serum of the cuprizone-induced multiple sclerosis in C57BL/6 mice

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Abstract

Introduction: Brain-derived neurotrophic factor (BDNF) and tumor necrosis factor- α (TNF α) are two critical factors in multiple sclerosis (MS). The aim of the present study is comparing the crosstalk between BDNF and TNF α in the brain versus serum and their effects on recovery of C57BL/6 mice demyelination in cuprizone model.

Methods: Fifteen C57BL/6 mice 6-week-old in three different groups were used: control (standard rodent chow), cuprizone-exposed1 (CPZ1; 0.2% cuprizone feeding for 5 weeks) and cuprizone-exposed2 (CPZ2; 0.2% cuprizone feeding for 5 weeks followed by 2 weeks of cuprizone withdrawal). To prove MS induction and cognitive behavioral impairment, Y-maze test and histological studies with luxol fast blue staining were done. The levels of BDNF and TNF α in the brain and serum were measured by ELIZA kits.

Results: In the present study, Y-maze test demonstrated that MS significantly impaired the cognitive behavior in both CPZ groups compared to control group. Demyelination in corpus callosum (CC) significantly was higher in the two CPZ groups relative to control group. The brain levels of BDNF significantly decreased while the brain levels of TNF α significantly increased in both CPZ groups compared to control group. Serum levels of BDNF and TNF α significantly increased in CPZ1 group compared to control group.

Conclusion: According to the present results, cuprizone-induced MS caused to an extensive demyelination in CC, which led to impaired cognitive behavior. Moreover, brain and serum levels of BDNF and TNF α as well as their crosstalk were affected by cuprizone exposure.

Keywords:

BDNF;
TNF α ;
Multiple sclerosis;
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Introduction

Multiple sclerosis (MS) is an autoimmune chronic inflammatory disease of the central nervous system (CNS) that characterized by the presence of pathogenic mononuclear cells and autoreactive T

cells which lead to demyelination as well as to axonal pathology. However, these cells also can be involved in neuroprotective processes by different pathways including the release of neurotrophic factors (Caggiula et al., 2005; Patanella et al., 2010). It has been reported that there are interactions between inflammatory and neurotrophic factors that could play

a specific role in MS (Patanella et al., 2010). Investigations showed that TNF_{α} as a pro-inflammatory cytokine, is the principal mediator in immune defense as well as the development and maintenance of the immune system, and its level elevates in a variety of the CNS disorders such as MS by secretion from autoreactive T cells, microglia and astrocytes (Lim and Constantinescu, 2010). This factor can exhibit both neuroprotective and neurodegenerative properties (Saha et al., 2006). Moreover, it can significantly alter the levels of a brain neurotrophic factor (BDNF) (Patanella et al., 2010). One of the brain neurotrophin families that most widely distributed in the CNS is the BDNF. Multiple cell types including CNS neurons and glia, B and T lymphocytes and monocytes in blood can express it (Caggiula et al., 2005; Saha et al., 2006; Klein et al., 2011). This factor participates in neuronal growth and maintenance, as well as in different aspects of activity-dependent synaptic physiology (Saha et al., 2006). Some investigations showed that BDNF is mainly implicated in clinical recovery and it has neuroprotective effects during physiological and pathological conditions of the brain (Saha et al., 2006; Patanella et al., 2010). According to some evidence, BDNF can cross the blood–brain barrier in mice. However, it is uncertain whether blood BDNF levels can reflect brain BDNF levels across species (Klein et al., 2011). There are different results on the interaction and correlation between TNF_{α} and BDNF in the brain and serum in various species of animals (Saha et al., 2006). In the present study, for MS induction determination, two procedures were used: 1- Y-maze test, was used to investigate the impairment of spatial working memory and cognitive behavior in the MS model of C57BL/6 mice, 2- luxol fast blue staining, is commonly used to observe myelin under light microscopy (Tsunoda et al., 2003). There are some investigations which used this staining for confirming the myelination (Boretius et al., 2012; Basoglu et al., 2013). On the other hand, the cuprizone mouse model allows the investigation of the complex molecular mechanisms behind nonautoimmune-mediated demyelination and spontaneous remyelination in animal MS model (Praet et al., 2014). At least in our search we have not found any direct report on the comparison between BDNF and TNF_{α} crosstalk in the brain versus serum of C57BL/6 mice cuprizone model of

MS. The evaluation of BDNF and TNF_{α} in other studies was not simultaneously in the brain and serum of cuprizone model of MS. Therefore, in the present study the main goal was to investigate the crosstalk between BDNF and TNF_{α} in the brain versus serum of the cuprizone-induced MS in C57BL/6 mice. Another goal of this study was to determine whether the spontaneous remyelination in the corpus callosum (CC) can be done in short time (two weeks) after cuprizone withdrawal or not.

Materials and methods

Animal

Fifteen adult male C57BL/6 mice (6-weeks old, 18 to 21g) (Xu et al., 2010) were purchased from Pasteur Institute (Karaj, Iran). Research and animal care were approved by the review board for the care of animal subjects of the district government (Shiraz, Iran). The mice were housed in a temperature 20–24°C, 12-hour light-dark cycle and humidity-controlled (Pfeifenbring et al., 2015).

Experimental design

Male C57BL/6 mice were randomly assigned into the 3 groups (n=5) after acclimatization for 14 days (Kashani et al., 2017): 1- control, standard rodent chow; 2- cuprizone-exposed 1 (CPZ1), 0.2% cuprizone feeding for 5 weeks and 3- cuprizone-exposed 2 (CPZ2), 0.2% cuprizone feeding for 5 weeks followed by a recovery phase of 2 weeks with a cuprizone-free diet (Fig. 1). All animals had access to water and food *ad libitum*. Cuprizone (Sigma-Aldrich, Germany) mixed in powdered standard rodent for a total of 5 weeks to induce reproducible patterns of demyelination of the CC (Hibbitts et al., 2009). Induction of MS were determined by Y-maze test in control and CPZ1 groups instantly after the removal of cuprizone, and then the mice were sacrificed for histological and biochemical analysis. In CPZ2 group, after 5 weeks of cuprizone feeding, they were fed normal chow for a subsequent 2 weeks period to allow progression of spontaneous remyelination and then after Y-maze test, the mice were sacrificed for histological and biochemical analysis (Song et al., 2005; Hibbitts et al., 2009).

Y-maze test

This task was performed as described in the previous

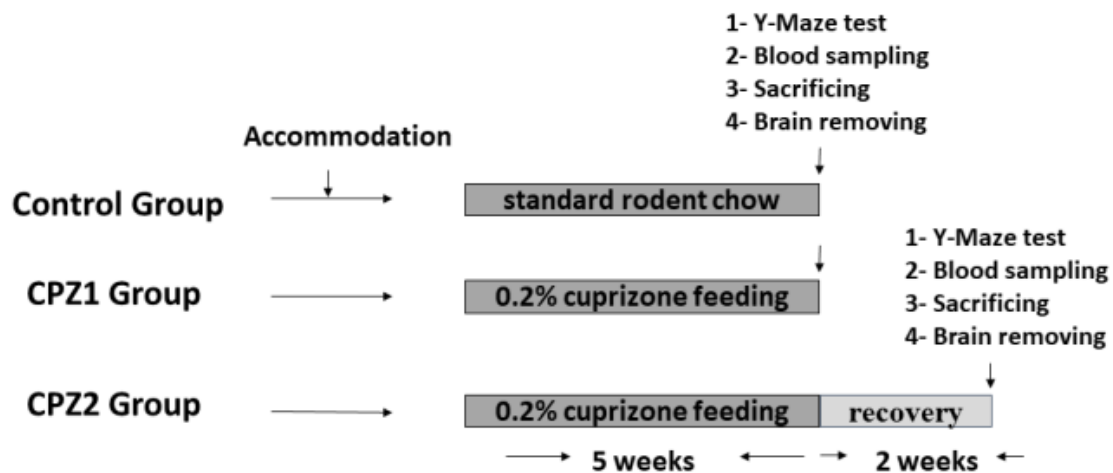


Fig.1. Experimental design paradigm.

study (Torkildsen et al., 2008; Xu et al., 2010; Yan et al., 2015). Each mouse was placed at the end of one arm (34cm long, 6cm wide and 14.5cm deep and labeled A, B or C) of a symmetrical Y-maze and allowed to move freely through the maze during an 8-min session. The total number and sequence of arms entries was recorded manually during the sessions. Actual alternation was the entries into the three arms on consecutive occasions. Finally, the maximum alteration was the total number of arm entries minus two. The calculation of the percentage of alteration was as following: Percentage of alteration = $\frac{\text{actual alteration}}{\text{maximum alteration}} \times 100$. (Torkildsen et al., 2008; Xu et al., 2010; Yan et al., 2015).

Histopathology

Mice were deeply anesthetized and sacrificed then whole brain was quickly removed, cerebrum and cerebellum were separated and cerebrum dissected into equal right and left hemisphere on ice. One of these hemispheres was fixed in 10% neutral buffered formaldehyde solution for 72 hours and after routine tissue processing, tissues were embedded in paraffin. For tissue analysis, 5 μ m coronal serial sections were prepared by a microtome (Xu et al., 2010; Wergeland et al., 2011). They were stained with luxol fast blue in order to assess the degree of demyelination in CC. Sections were examined by Olympus BX51 microscope using 2.5 \times and 10 \times objective lenses and pictures were taken by the Olympus DP 20 digital camera attached to the microscope (Olympus, Tokyo, Japan) (Basoglu et al., 2013).

Quantification of images

Brain images were quantified using the free Java image processing software ImageJ. For assessment of myelin levels through optical density (OD), five coronal sections from each animal were chosen for OD measurement. In each image, the cross sectional area of CC was calculated by imageJ as previously described (Bernardes et al., 2013; Wang et al., 2013; Madsen et al., 2016).

Measurement of brain and serum BDNF and TNF α

In all mice after deep anesthesia for serum preparation, whole blood samples were taken from their hearts. Then collected in anticoagulant-free tubes and kept at room temperature for 10-20 min. According to the protocol, the samples were centrifuged (2000-3000 RPM) for 20 minutes, the supernatants were collected and aliquoted and stored at -80 $^{\circ}$ C. Moreover, as soon as sacrificing and dissecting animals, one of the brain hemispheres of each animals was frozen on dry-ice and quickly transferred to -80 $^{\circ}$ C freezer and stored. For preparation of brain tissue, the brain was homogenized (1:5, w/v) with PBS (pH: 7.4) thoroughly by sonication on the ice. The homogenates were centrifuged (at 2000- 3000 RPM, 4 $^{\circ}$ C) for 20 minutes. The supernatant was carefully collected, aliquoted and stored at -80 $^{\circ}$ C (Elfving et al., 2010). Brain and serum BDNF and TNF α concentrations were assayed directly using specific ELISA kits in accordance with the manufacturer's instructions (Bioassay Technology Laboratory, E0013Mo and E0117Mo, Shanghai Crystal Day

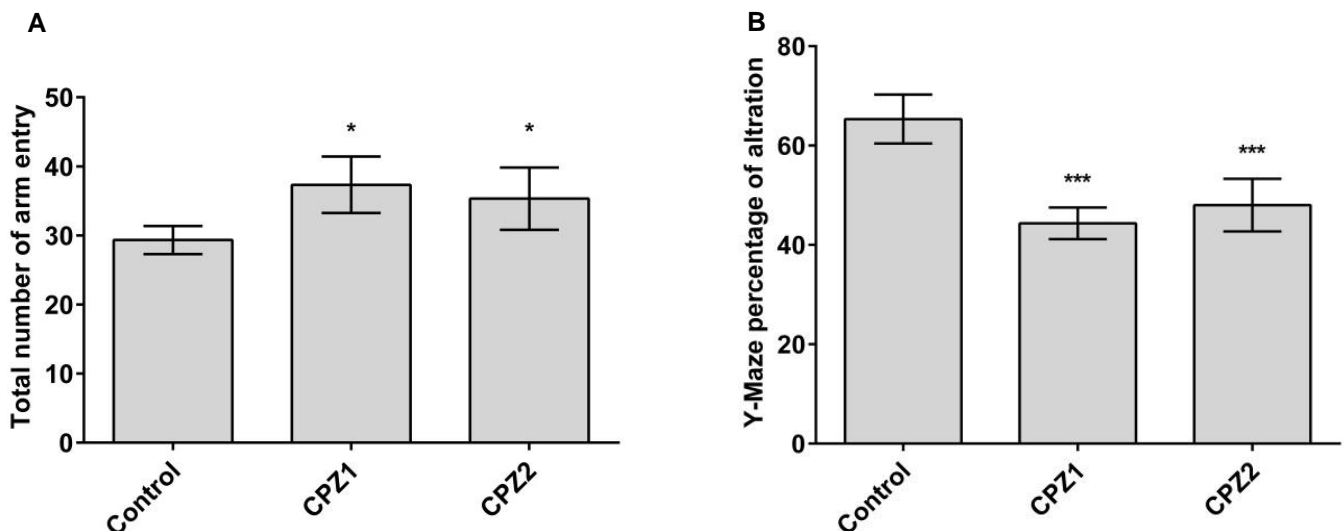


Fig.2. Effect of cuprizone induced MS on (A) total number of arm entry; (B) percentage of alteration immediately after cuprizone withdrawal (CPZ1) and 2 weeks after cuprizone withdrawal (CPZ2) in Y-maze test. * $P<0.05$ and *** $P<0.001$ significant compared to control group. Data were shown as mean \pm SEM.

Biotech Co., Ltd. China).

Statistical analysis

Data was analyzed on SPSS Statistics version 21 (IBM Co.). Multiple comparisons between the control and the two cuprizone-exposed groups were performed using one-way ANOVA followed by the Tukey as post-hoc test. The significance level was considered at $P<0.05$. All data were expressed as the mean \pm SEM (Bernardes et al., 2013).

Results

Behavioral responses

In Y-maze test, the alternation behavior significantly decreased in both the CPZ1 [$F(2)=13.3$; (CPZ1: $P<0.001$ and CPZ2: $P<0.001$)] groups compared to control group (Fig. 2A). The number of entries to each arm was significantly [$F(2)=1.88$; ($P<0.05$)] higher in the CPZ1 and CPZ2 mice than in the control mice. While, the alternation behavior of CPZ2 group [$F(2)=1.88$; ($P>0.05$)] seemed to be higher and the number of entries to each arm to be lower relative to CPZ1 group but did not reach significant levels, suggesting the 2weeks recovery period was not sufficient to improve the behavior and the motor activity of mice (Fig. 2B).

Demyelination analyses

As expected, in the animals exposed to cuprizone, the myelin was significantly reduced in the both CPZ1

and CPZ2 [$F(2)=825$; ($P<0.001$)] groups compared to control group. Moreover, CPZ2 group showed a significant lower demyelination ($P<0.05$) compared to CPZ1 group, suggesting 2 weeks recovery period could be effective on remyelination with significant levels in CC (Fig. 3). The blue color of CC was reduced in two CPZ groups relative to control group (Fig. 3)

BDNF and TNF α measurements in the brain and serum

To investigate the role of neurotrophic factors and inflammatory mediators in the cuprizone-induced MS and recovery period, BDNF and TNF α concentration were evaluated in the brain tissue and serum samples from the CPZ1, CPZ2 and control groups. According to the present data, BDNF levels were significantly lower in the brain of the CPZ groups [$F(2)=13.3$; (CPZ1: $P<0.01$ and CPZ2: $P<0.01$)] compared to control group (Fig. 4A). Serum BDNF levels were significantly higher [$F(2)=18.08$; ($P<0.01$)] in CPZ1 compared to control, but there is no significant difference between CPZ2 and control groups. Serum BDNF levels in CPZ2 were significantly lower [$F(2)=138$; ($P<0.05$)] than CPZ1 (Fig. 4B).

According to present data the TNF α levels in brain tissues of CPZ groups were significantly higher [$F(2)=179$; ($P<0.01$)] than control group. Whereas, there was no significant difference in the levels of the brain TNF α in the CPZ2 group compared to CPZ1

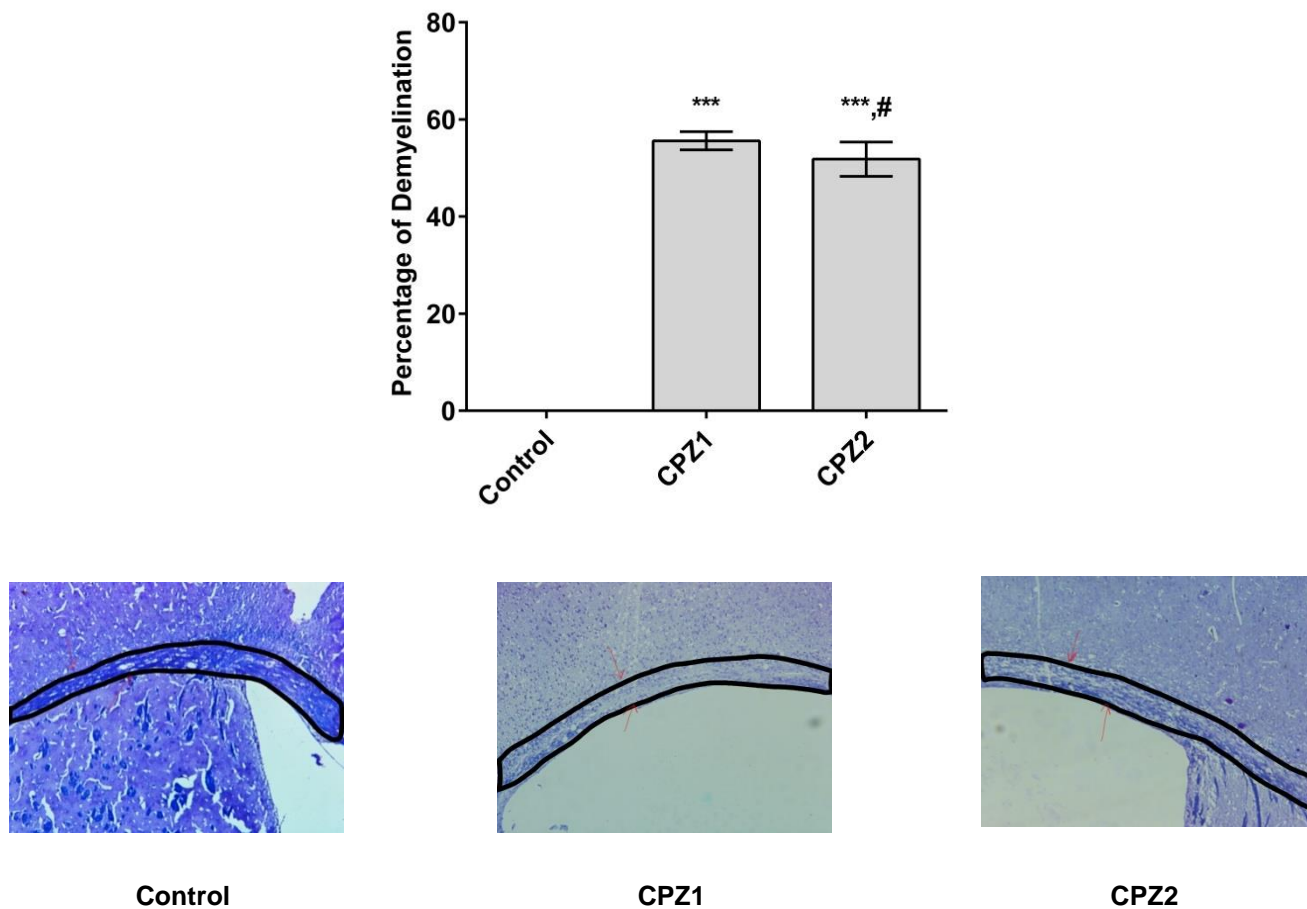


Fig.3. Effect of cuprizone induced MS on percentage of demyelination cross sectional area of corpus callosum (CC) immediately after cuprizone withdrawal (CPZ1) and 2 weeks after cuprizone withdrawal (CPZ2); and microphotograph sections of CC that represented the rate of demyelination, blue color represent the myelination and black line and red arrow represent the border of CC in hemisphere (magnification 10×10). ****P*<0.001 significant compared to control group, #*P*<0.05 significant compared to CPZ1. Data were shown as mean±SEM. CPZ1: cuprizone-exposed 1, CPZ2: cuprizone-exposed 2.

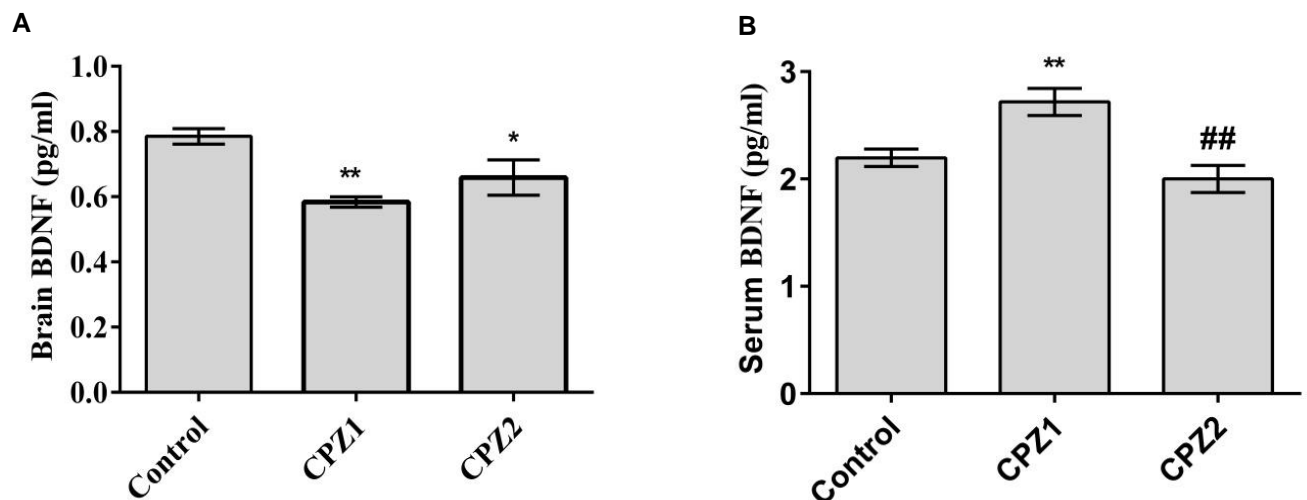


Fig.4. Effect of cuprizone induced MS on brain (A) and serum (B) BDNF immediately after cuprizone withdrawal (CPZ1) and 2 weeks after cuprizone withdrawal (CPZ2). **P*<0.05 and ***P*<0.01 significant compared to control group, ##*P*<0.01 significant compared to CPZ1 group. Data were shown as mean±SEM. CPZ1: cuprizone-exposed 1, CPZ2: cuprizone-exposed 2.

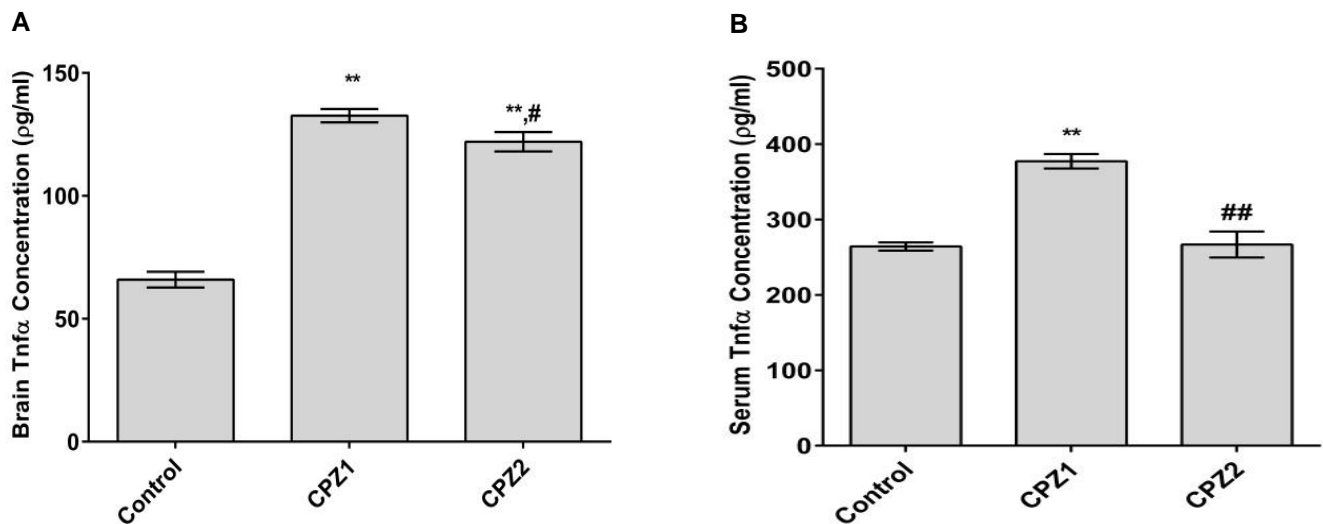


Fig.5. Effect of cuprizone induced MS on brain (A) and serum (B) TNF α immediately after cuprizone withdrawal (CPZ1) and 2 weeks after cuprizone withdrawal (CPZ2). ** P <0.01 significant compared to control group, # P <0.05 and ## P <0.01 significant compared to CPZ1 group. Data were shown as mean \pm SEM. CPZ1: cuprizone-exposed 1, CPZ2: cuprizone-exposed 2.

group (P >0.05, Fig. 5A). Moreover, present results revealed the serum TNF α levels in CPZ1 group were significantly [F(2)=63.6; (P <0.01)] higher than control group. Serum TNF α levels in CPZ2 were significantly lower [F(1)=0.05; (P <0.01)] than CPZ1 (Fig. 5B).

According to these results, BDNF and TNF α levels altered in parallel in the serum samples of the CPZ groups, while these factors inversely altered in the brain tissues of the mentioned groups.

Discussion

Our results showed that the intake of 0.2% cuprizone for 5 weeks led to distinct and significant demyelination in the CC of the two CPZ groups. Consistent with our data, the previous studies showed a significant demyelination after 3 weeks. Kipp et al. reported that remyelination was occurred spontaneously following cuprizone removal; therefore, the pathological and histological changes gradually revert (Kipp et al., 2009; Zendedel et al., 2013; Vega-Riquer et al., 2019). As we have found, significant but not completed remyelination was occurred after 2 weeks of the cessation of cuprizone treatment. According to the other investigations, in this model during acute demyelination partial remyelination occurs between week 3 and 5 (Kipp et al., 2009). Therefore, present study suggested two weeks of recovery time was not sufficient for complete remyelination and the various factors, involving in the remyelination, needed more time to

accomplish this process.

In the present study, Y-Maze test was used to count the arm entries and alternations for estimating the spatial working memory in the animals. As expected, the two groups with cuprizone-induced demyelination had significantly lower percent of spontaneous alternation and the higher total number of arm entries than control group in Y maze test. According to the previous studies, myelin is an important component for the function of neuronal circuits properly; therefore, abnormalities of the myelin sheath may contribute to the deficiency of neuronal function, and myelination is actively involved in the plasticity of neuronal circuits in adult animals and essential for cognition and motor skills (Li et al., 2015). Some reports showed that in cuprizone-induced MS, mice exhibited global and widespread demyelination in the brain and spontaneous remyelination was occurred following cuprizone removal. These mice displayed abnormal behaviors, cognitive deficits and impairments of spatial working memory that are believed to be a direct result of demyelination (Xu et al., 2009; Li et al., 2015). Consistent with the previous evidence, present results revealed impaired spatial working memory in the both CPZ1 and CPZ2 groups, suggesting this period time was not sufficient to improve spatial working memory and behavioral deficits significantly.

There are some studies in a few species of animals with different or sometimes contradictory results that investigated the levels of inflammatory cytokines like

TNF α and neurotrophic factors like BDNF which involved in the neurodegenerative disorders such as MS. However, we have not found any direct report on the crosstalk between BDNF and TNF- α in the brain versus serum of C57BL/6 mice cuprizone model of MS. According to the present investigation, the brain BDNF levels were significantly lower in the CPZ groups compared to the control group. In addition, the brain TNF α levels were significantly higher than the control group.

Several studies showed histopathological changes in the brain and serum of the cuprizone model of MS including: 1) astrogliosis as well as astrocyte reactivity that is prolonged and may have an important role during recovery period (Hibbitts et al., 2012). Some evidences were demonstrated that astrocytes by secreting different factors such as BDNF and TNF α are key regulator; they provide a signal to recruit microglia and oligodendrocyte progenitor cells toward lesions and facilitate each step of de- and re-myelination of MS (Skripuletz et al., 2013; Domingues et al., 2016); 2) loss of oligodendrocyte, which is directly affected and perturbed by toxic effects of cuprizone (Hibbitts et al., 2012; Clarner et al., 2015; Domingues et al., 2016) and 3) microgliosis and activated microglia that expand, migrate and accumulate within the lesions site due to some factors like TNF α for contributing to oligodendrocyte and myelin damage during demyelination, as well as for removing damaged myelin sheaths to start remyelination (Skripuletz et al., 2013; Domingues et al., 2016). Besides, these cells can directly affect astrocytes through expressing TNF α (Allan and Rothwell, 2001).

Evidence indicated that astrocytes exert both deleterious effects during early stage of CNS damage and protective effects in the future (Skripuletz et al., 2013; Clarner et al., 2015). Moreover, TNF α through its two receptors that are exist on the astrocytes, acts as an immunomodulatory cytokine. TNFR1 mediating primarily inflammation, pathological processes and pro-apoptotic functions that are important for the onset of MS, and TNFR2 inducing cell survival, resolution of inflammation, immunity and proliferation of oligodendrocyte progenitor cells and re-myelination (Dong et al., 2015; Madsen et al., 2016). According to the previous results, at the later stages of MS, TNF α activates TNFR-2 on astrocytes which is required for up-regulation of BDNF production with an anti-

inflammatory role stimulating proliferation and differentiation of oligodendrocytes and myelin protein synthesis (Li and Ransohoff, 2008; Praet et al., 2014). Besides, all glia including astrocyte, microglia and oligodendrocyte as well as immune cells (B and T lymphocytes and monocytes), through BDNF production as a growth factor caused to decrease production and release of TNF α (Gielen et al., 2003; Caggiula et al., 2005; Bernardes et al., 2013; Clarner et al., 2015).

There are some investigations reported that cuprizone demyelination model of MS, led to decrease of the expression and levels of BDNF in the CC. Therefore, the animals exhibited a more severe loss of myelin in the CC. However, it is well known that glial cells increase expression of BDNF following injury, but the demyelinating lesion itself elicits a reduction in the brain- tissue BDNF levels through the various signals of lesion (VonDran et al., 2011; Fulmer et al., 2014). In contrast, it has been reported that pro-inflammatory cytokines, like TNF α , are significantly up-regulated and increased by microglia, macrophage and astrocyte in the MS lesions (Gielen et al., 2003; Caggiula et al., 2005; Schmitz and Chew, 2008; VonDran et al., 2011; Dong et al., 2015). Moreover, it was indicated that TNF α levels increase during MS in parallel with clinical course (Schmitz and Chew, 2008). These evidences were consistent with our results that suggested the crosstalk between BDNF and TNF- α in the brain of cuprizone mice model caused to lower levels of BDNF and higher levels of TNF- α compared to control mice.

At first in cuprizone-exposed mice, there were higher levels of the brain TNF α which mediated demyelinating processes through TNFR1 signaling to decrease brain BDNF production that occurred during cuprizone treatment; however, at the later stage, TNF α by TNFR2 mediated remyelination processes caused to increase brain BDNF levels. On the other hand, higher brain BDNF levels caused to modulate and decreased the brain TNF α levels which could occur during 2 weeks recovery as the present study showed significant higher levels of the BDNF and lower levels of the TNF α in the brain tissue of CPZ2 group compared to the CPZ1 group. According to the present study and some other investigations, spontaneous re-myelination were initiated following cuprizone withdrawal and it can be completed after 5-

6 weeks (Kipp et al., 2009; Vega-Riquer et al., 2019). Hence, after 2 weeks of recovery, remained lesions still caused to prevention of the BDNF production within the normal range in the brain; therefore, modulation of the brain TNF- α levels were insufficient.

In addition, present results revealed that the serum levels of BDNF and TNF- α in CPZ1 group were significantly higher in comparison to control and CPZ2 groups. Researchers reported TNF- α up-regulates BDNF that may be responsible for neurotrophic activity of the cytokine (Saha et al., 2006). On the other hand, the immune system cells that produce and release neurotrophins like BDNF can express neurotrophin receptors that seems to be the target of neurotrophin autocrine and paracrine actions (Caggiula et al., 2005). So, BDNF can modulate and decrease TNF- α as well as itself through these cells. It was reported that BDNF can play a protective role by regulating the release of pro-inflammatory cytokines like TNF- α and modulating the cytokines expression from pro-inflammatory to anti-inflammatory as well as blocking the deleterious effects of TNF- α on CNS cells during inflammation for suppressing the new lesions formation (Gielen et al., 2003; Caggiula et al., 2005; VonDran et al., 2011). According to these reports, at first, the higher levels of TNF- α in the serum caused to up-regulation of serum BDNF levels during cuprizone treatment; however, at the later stage, increased levels of serum BDNF caused to the down-regulation and decreased serum levels of TNF- α as well as BDNF, as we demonstrated after 2 weeks recovery in CPZ2 mice. Consistent with these data, present results suggested that the crosstalk between BDNF and TNF- α leads to significantly increased BDNF and TNF- α levels in the serum after cuprizone treatment, and 2 weeks of recovery period was a sufficient time for BDNF and TNF- α to regulate the production and release of each other in the serum.

Conclusion

According to the present study, demyelination of cuprizone mice model of MS could be recover after 2 weeks; however, it was not complete. Therefore, impairment of spatial working memory with no significant improvement of behavioral deficits was still observed after the 2 weeks of the recovery time.

Moreover, the crosstalk between BDNF and TNF- α were affected in the brain and serum of the cuprizone-induced MS in C57BL/6 mice. BDNF and TNF- α levels altered in parallel in the serum samples of the MS groups; while, these factors altered inversely in the brain tissues of the mentioned groups. In addition, a short term of recovery as long as 2 weeks was sufficient to revert the serum BDNF and TNF- α levels within their normal ranges but not a sufficient time to improve the brain BDNF and TNF- α levels, within the normal in this model.

Acknowledgments

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Conflict of interest

All the authors confirm that, there is no financial or other relationship, which could cause a conflict of interest.

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