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Pretreatment Effect of a 6-week Swimming Training protocol along with Vitamin D administration on the brain levels of BDNF, TNF-α and IL-10 in Rats Model of EAE

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ABSTRACT:

Experimental Autoimmune Encephalomyelitis (EAE) is an appropriate model for investigating the inflammation indices in Multiple Sclerosis (MS). The aim of this study was to evaluate the pretreatment effect of 6-week forced swimming exercise alongside vitamin D supplementation on levels of cytokines and brain-derived neurotrophic factor (BDNF) in rats with EAE. Swimming exercise was performed for 5 d/week over a period of 6 weeks. The swimming training program initiated with 30 minutes in the first week and then by adding 5 min daily it reached 60 min in the second week. The overload was exerted by adjusting the workload through increasing the water flow speed. EAE was induced at the end of the sixth week of training. The vitamin D was injected 2 weeks (every two days) before inducing the EAE model. The animals were sacrificed 14 days after EAE induction. Disease progress was evaluated daily. Statistical analysis was done using a one-way analysis of variance. The results of ELISA test indicated that swimming exercise alongside vitamin D in Lewis female rats with EAE led to lower concentrations of TNF-α ($p<0.0001$). However, there was not observed a significant change in IL-10 ($p<0.05$) and BDNF ($p<0.05$). The results of current study suggest that this protocol would possibly help neuroprotection through confronting the EAE-stimulated inflammation. However, there are more studies required for discovering the mechanisms through which forced swimming exercise alongside vitamin D exert their neuroprotective roles.

KEY WORDS BDNF, experimental autoimmune encephalomyelitis, IL-10, TNF-α, vitamin D, swimming training

INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory immune system disease. The exact cause of its incidence is rather unclear. This debilitating disorder causes injuries to central nervous system (CNS), spinal cord, brain stem and some parts of the brain white matter leading to impairments in neurotransmission from these regions [1].

Experimental autoimmune encephalomyelitis (EAE) is a reliable model for studying inflammatory signaling in MS. In this model, the T cells of animals are directly exposed to myelin antigens stimulating demyelination [2]. It has been demonstrated that vitamin D alters the immune responses [3]. In other words, the insufficiency of this vitamin increases the susceptibility of the body to MS occurrence [4]. Vitamin D can be supplied from diet, supplements and through endogenous production. However, only 30% of the body's total vitamin D requirement is provided through diet. Thus, the main supplier of this vitamin is the exposure to sunlight or through the use of supplements [5]. Previous works has suggested that vitamin D could prevent the occurrence of the disease, providing a rationale for continuing research in this area [6]. Several experimental studies have shown that using vitamin D results in reduction of immune system inflammation [7]. The immune and the nervous systems are characterized by striking parallels. complemented by a complicated assembly of factors that induce cell growth and differentiation. In the immune system, these factors are identified as cytokines and in the nervous system they are called neurotrophic factors [8].

Cytokines can be divided into pro-inflammatory and anti-inflammatory isoforms. MS is an autoimmune disease where an imbalance between pro- and anti-inflammatory cytokines plays a pivotal roles in lesion incidence and deterioration of symptoms [9]. Among these cytokines, TNF-α is a pro-inflammatory cytokine that can activate immune system-mediated inflammation in CNS [11]. On the other hand, IL-10 is an anti-inflammatory cytokine which is related to the remission period of MS [10]. It is now revealed that brain-derived neurotrophic factor (BDNF) is expressed in immune cells [12]. BDNF production have been shown in infiltrating immune cells in the experimental models of CNS injury and respiratory inflammation [13]. Clinically, the roles of neurotrophins in MS is complicated. Studies have often shown paradoxical results in BNDF concentrations during MS treatment period remained unchanged [14], decreased [15] or increased [16].

Exercise-based protocols for animal models encompass wheel running, treadmill running, resistance exercises and swimming training. Although both voluntary and forced exercises are suggested as two potential approaches for neuroprotection, it seems that forced exercise, because of changing the brain metabolism, is more effective than voluntary exercise in promoting neuroprotection [17]. As a result, using forced exercise as a pre-treatment protocol prior to the induction of EAE in animal models sounds to be a better approach to study the effects of physical activity prior to the onset of disease. Furthermore, by manipulating the intensity, duration and frequency of training, an appropriate protocol can be identified. Based on previous studies, it seems that a training period of more than 3 weeks is mandatory for increasing adaptability with attenuated stress responses [18]. Also, in a comparison between 6-week forced treadmill running and swimming training on the reduction in injuries of motor neurons and oligodendrocytes in ALS model, the swimming training was more effective [19]. Bernardes and colleagues (2014) have studied the effects of 6-week forced swimming training on the concentrations of IL-10, IL-1β, TNF-α, IL-6 cytokines and BDNF in the brain and the spinal cord tissues in rats' model of EAE. The results of their study have shown that there was a considerable increase in IL-1β in the brain, TNF-α, IL-6 in the spinal cord and BDNF in both tissues [20].

All the while, there are not any studies investigating the effect of combining swimming exercise and vitamin D administration in rat model of EAE. Therefore, we hypothesized that preconditioning with forced swimming and vitamin D supplementation can protect brain tissue against inflammatory cytokines. Thus, the purpose of this study was to investigate the effects of pre-conditioning with forced swimming training along with vitamin D injection in EAE models of Lewis female rats on the IL-10, TNF-α and BDNF levels.

METHODS

Forty, six-week old, female Lewis rats were provided from Razi Vaccine and Serum Research Institute of Karaj, Iran. Animals were randomly divided equally into 8 groups: control healthy (C-H), control MS (C-EAE), control MS vitamin D (C-EAE-VD), exercise MS vitamin D (EX-EAE-VD), exercise healthy vitamin D (EX-H-VD), vehicle (V), exercise MS (EX-EAE) and exercise healthy (EX-H). During the study period, animals had free access to water and nutrition. The overall design of this research is presented in Figure 1. The use of animals for this protocol was reviewed and approved by the care and use committee of the University of Mazandaran.

Swimming training protocol

The training groups were subjected to once daily swim training, 5 days a week for 6 weeks. The training protocol was initiated with a 1 week ramping protocol with rats starting at 30 min of swimming on day 1 and increasing the duration of swimming by 5 min until 60 min was reached in week 2. Overload was conducted in weeks 2-6 by modulating the speed of the water flow. In the training period, the speed of the water flow reached 20 lit/min from 7 lit/min, while the duration remained at 60 min.

EAE induction

Rats were immunized using a combination of guinea pig spinal cord and complete Freund adjuvant (Sigma F5881, CFA) containing oil-in-

water emulsified mycobacteria. Spinal cords were taken out from anesthetized guinea pigs, placed in saline and were transferred to -40 °C for 25 min afterwards. The mycobacterium concentration in CFA was offset at 4 mg/ml [21]. At the end of the sixth week, the EAE model was induced (tail base). The assessment approach of disease intensity, which was conducted by a researcher blinded to the treatments beginning the eighth day continuously included: 0 = no clinical signs 1 = tail paralysis (or loss of tail tone), 2 = tail paralysis and hind-limb weakness, 3 = hind-limb paralysis and 4 = complete hind-limb paralysis and front- limb weakness [20].

Vitamin D injection

The treatment with vitamin D or sham injections started two weeks before EAE induction, and it continued for 2 weeks until the end of the training sessions [22]. EAE rats randomized to vitamin D treatment received 2 µg/BW vitamin D in 150 µl sesame oil every other day through intraperitoneal injections. In EAE control group rats, sham injections with 150 µl sesame oil were administered via intraperitoneal injections every other day.

Tissue preparation and measurements

Rats were anesthetized through intraperitoneal injection of Ketamine and Xylazine. After removing the whole brain from the skull, the brain was divided into right and left hemispheres, then the left hemisphere was quickly placed in liquid nitrogen and maintained in -80 °C until analysis. After homogenizing, samples were centrifuged for 12 mins at 12000 g BDNF concentrations were measured using Zell Bio commercial kit (Germany), and the TNF-α and IL-10 were measured by Diaclone commercial kit (France) using ELISA test according to the instructions of the kits.

For determining the normality of the data distribution in different variables, Shapiro-Wilk test was utilized where the significance level was $P \leq 0.05$. One-way analysis of variance (One-Way ANOVA) and the Tukey`s post hoc test were used, for analyzing the intergroup differences, at the significance level of $P \leq 0.05$. All the statistical analysis was performed using GraphPad Prism software (version 6.07).

STATISTICAL RESULTS

The purpose of this study was to assess the effects of exercise and vitamin D preconditioning on brain proteins associated with neurodegeneration and neuroprotection in an EAE model. We hypothesized that preconditioning with forced swimming and vitamin D supplementation can protect brain tissue against inflammatory cytokines.

All the animals successfully completed the training protocol. Therefore, all of them were employed for data analysis. EAE animals have shown more disability compared to healthy animals ($P<0.05$). No debilitating clinical symptoms have been observed among non-EAE animals. The first clinical sign of the disease showed up on the tenth day after inducing the EAE model. No significant differences were observed for body mass.

TNF-α

ANOVA revealed significant differences between the groups with respect to TNF-a concentrations in the brain ($F(7, 26) = 9.838$; $p<0.0001$). Post-hoc analysis revealed that C-EAE group had significantly greater TNF-alpha concentration compared to the C-H group ($p=0.0006$). Specifically, C-EAE group had significantly greater TNF-alpha compared to EX-EAE-VD ($p=0.007$), EX-H-VD ($p< 0.0001$) and V groups ($p< 0.0001$) (Figure 2). These results show that the combination of exercise and VD considerably decreased the concentration of this cytokine.

IL-10

Following the 6-week swimming training protocol, no significant difference was noticed in IL-10 concentration ($F(7, 28) = 0.8597$, $P = 0.5493$) (Fig. 3). IL-10 concentration in C-EAE-VD group showed a decremental inclination (359.1±64.89 pg/ml).

BDNF

There was no observed significant differences between the study groups regarding BDNF concentration ($F(7, 28) = 1.224$, $P = 0.323$) (Fig. 4). The BDNF concentration among the groups was as following: 1.316± 0.1074 ng/ml in C-H, 1.361± 0.1042 ng/ml in C-EAE, 1.227± 0.0482 ng/ml in C-EAE-VD, 1.227± 0.1447 ng/ml in EX-EAE-VD, 1.303± 0.07397 ng/ml in EX-H-VD, 1.217± 0.1140 ng/ml in V, 1.281± 0.08308 ng/ml in EX-EAE and 1.244± 0.09888 ng/ml in EX-H.

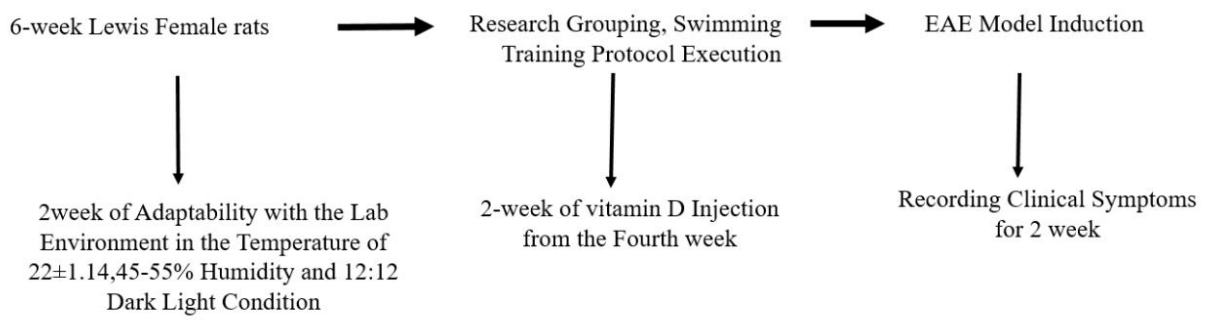

Figure 1. The overall design of the study

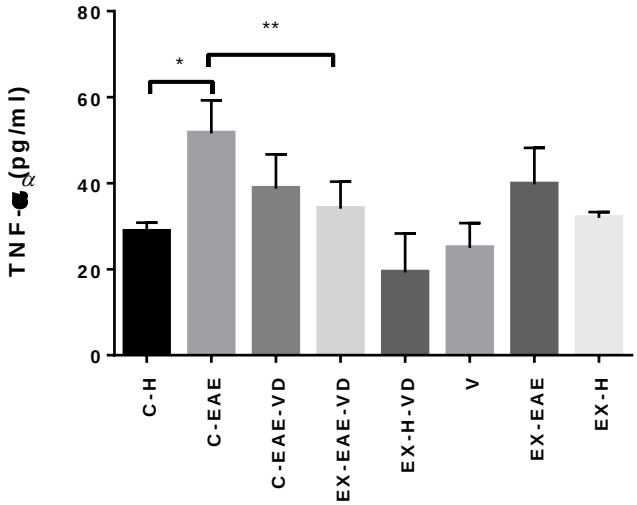

*Figure 2. Comparing the TNF-α levels in the brain: * significant difference between C-EAE with C-H (p=0.0006) **significant difference of C-EAE group with EX-EAE-VD (p< 0.007).*

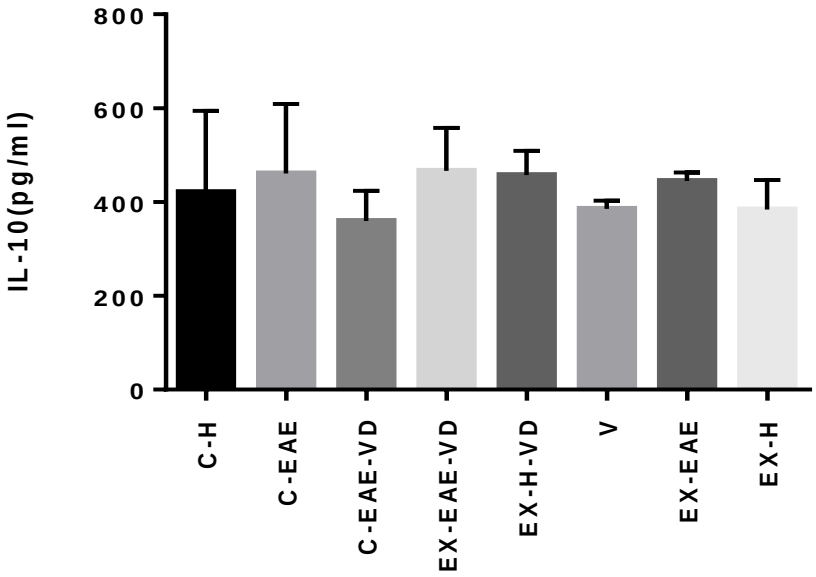

Figure 3. Comparing the levels of IL-10 in the brain tissue do not show significant differences among groups (P ≤ 0.05)

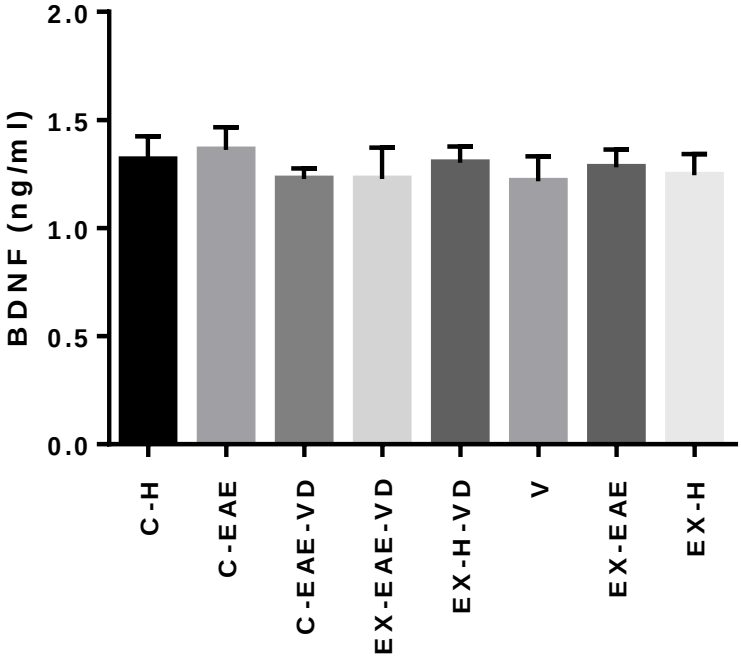

Figure 4. Comparing the BDNF levels of the brain between groups (P ≤ 0.05)

DISCUSSION

The purpose of this study was to investigate the neuroprotective effects of swimming training along with vitamin D supplementation on the brain BDNF and cytokines of the EAE model of Lewis female rats prior to disease onset. The results of this study revealed that swimming exercise in combination with Vitamin D supplemented preconditioning reduce TNF-alpha concentrations in the brain of EAE rats. These results are favorable despite no significant differences seen in the anti-inflammatory cytokine IL-10 or the neurotrophin protein BDNF. Bernardes and colleagues (2016) have investigated the effects of regular exercise pretreatment on clinical symptoms improvements derived from demyelination reduction and axonal injury in autoimmune encephalomyelitis. The results of their study demonstrated that long-term pretreatment over a period of 6 weeks improves clinical symptoms and reduces the intensity of pathological symptoms in EAE [19]. Bernardes and colleagues (2013) outcome support the results of our investigation and confirm that exercise can be effective in reducing pathology associated with EAE [20]. Nevertheless, others have reported that exercise could not decrease these symptoms which has been attributed to the inadequacy of training intensities and durations [23].

The study conducted on EAE shows that vitamin D prevents the progression of the disease [24]. Currently there has been a growing interest in using vitamin D as a regulatory factor of immune system in treating MS. The studies have demonstrated that vitamin D is efficacious in reducing the intensity of autoimmune diseases, particularly MS [5, 7]. But, the mechanism in which vitamin D exerts its effects is rather unclear. However, it is probable that vitamin D reduces the cellular infiltration that can be effective in controlling the disease [25], which is in accordance with our study. Also vitamin D can increase the secretion of anti-inflammatory cytokine, IL-10, from TCD4+ cells. They have shown that one reason behind the decrease of the secretion in the brain and spinal cord is the increase in apoptosis of infiltrated cells to CNS.

The results of our study have shown that there is not a significant difference in IL-10 levels following a swimming training program alongside vitamin D. IL-10 is a regulatory cytokine which is also observed during the remission period in EAE and MS patients. However, the decrease in TNF-α observed in this study was able to reduce the incidence of the disease symptoms. This finding is likely related to the effects of exercise along with vitamin D on the regulation and balance of cytokines in MS patients. Previous studies have shown that exercise is associated with the secretion of inflammatory cytokines, IL-4, IL-6, TNF-α and anti-inflammatory cytokine, IL-10 [26]. Interestingly, since the ratio between these IL-10 and TNF-α in MS patients is impaired, exercise as a modulator is able to restore this imbalance [25].

Other major finding of our study is that pretreatment with exercise or Vitamin D supplementation do not influence BDNF levels in the brain tissue of EAE model Lewis rats. In congruency with our results, Patel and White (2013), in a study investigating the effects of 10-day forced treadmill running on neurotrophic factors in Lewis rats with EAE, did not observe any significant differences in the brain BDNF and clinical symptoms between forced treadmill running and control groups [27]. On the other hand, Castellano and White have revealed a reduction in BDNF serum levels following a 4-week training protocol, which returned to the baseline in the eighth week in just the MS group [28]. Similar results have been reported in healthy animal models [29]. Similar, Zoladz and colleagues (2008) have attributed the increase in plasma BDNF responses to exercise [30]. Therefore, with regard to these paradoxical results, more studies are required to investigate the potential effects of swimming training program on BDNF in rats' model of EAE. In majority of studies, BDNF level returns to its initial level during 10 to 60 min after exercise which shows the high rate of BDNF removal from blood circulation after intercepting the exercises. Castellano and White [28] and Yarrow et al. [31] have reported a significant decrease in BDNF levels below pre-exercise baseline 2 to 3 h after exercise. In MS patients and healthy people, the response of BDNF to exercise seems to be resulted from BDNF secretion to blood circulation and also from increasing the tissue uptake.

The findings of this study have shown that using vitamin D alongside swimming exercise can reduce pro-inflammatory TNF-alpha concentrations. Measurement of BDNF levels did not show any changes. Nevertheless, the unchanged levels of IL-10 and BDNF have demonstrated that the neuroprotective effect of swimming exercise alongside vitamin D is not exerted through anti-inflammatory cytokines and neurotrophic factors, and therefore there should be other mechanisms involved. Thus, for unravelling the responsible mechanisms for this neuroprotective effect more studies are required.

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