**INTRODUCTION**

Myelin sheath around the axon is seriously important for fast conduction of electrical impulses in the central nervous system (CNS) (Miron, Kuhlmann, Antel, 2011). Demyelination of the axon is associated with repair processes called oligodendrocyte-induced remyelination. Animal studies have proved that remyelination happens during the four weeks of induced demyelination, although it is not always parallel to the improvements in behavioral performances (Patrikios et al., 2006; Shi et al., 2015). Focal injection of chemical toxins like EB into hippocampus is a method to cause demyelination through oligodendrocyte cell death (Goudarzvand et al., 2016; Goudarzvand et al., 2010). In addition, the effects of EB on demyelination and apoptosis pathway activation of hippocampus have been shown in molecular study in which anti-apoptotic effects of vitamin D₃ were investigated (Goudarzvand et al., 2016).

**Keywords:** Vitamin D₃. Nitric oxide concentration. Spatial memory. Ethidium bromide. Morris water maze.

Vitamin D₃ mediated spatial memory improvement through nitric oxide mechanism in demyelinated hippocampus of rat

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Studies have revealed beneficial role of vitamin D₃ in neuro-cognitive function. There is also supporting evidence on the involvement of nitric oxide (NO) in the neuro-protective action. However, its over production could contribute to brain disorders. In this study, demyelination was induced by ethidium bromide (EB) injection into the right side of the hippocampus area of male rats. Vitamin D₃ was administered to rats for 7 and 28 days prior to behavioral experiments using Morris water maze (MWM). Travelled distance, time spent to reach the platform, and time spent in target zone, were considered for learning and spatial memory evaluation. Nitrite oxide (NO₂⁻) concentration was measured as an indicator for nitric oxide production. The time spent to reach the platform and the travelled distance were decreased significantly by 28 days of vitamin D₃ administration (compared to 7 days experiment). Time spent in target quadrant was significantly lowered by administered vitamin on day 28. Therefore, considering a number of studies that have shown the effect of vitamin D₃ on cognition, these findings could support their potential effect. Besides, nitric oxide concentration significantly differed in 28 days of vitamin D₃ treated group compared with the groups treated with EB or 7 days of vitamin D₃.

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yet cleared. There are some contradictory results that vitamin D₃ has dual effect on the cognition (Brouwer-Brolsma et al., 2014).

Inconsistent conclusions and rather complex mechanistic explanations are consolidated for learning and memory impairment through antioxidative pathway and nitric oxide synthase (NOS) activity. Pitsikas (2015) is emphasized that both NO donors and NO synthase inhibitors are involved in object recognition memory. A number of studies reported that NO has the memory improvement effect (Babaei et al., 2012; Garry et al., 2015), however, researchers have reported that increased NOS activity and NO production in different area of the brain like hippocampus, are associated with memory deficits (Najafi et al., 2013; Wiley, Willmore, 2000; Yu et al., 2013).

Limited studies have been performed on the role of antioxidants in the hippocampus grey matter through behavior evaluation by animal modeling of EB induced demyelination. Consequently, this package was conducted to evaluate the antioxidative aspects of administration of vitamins D₃ through NO involvement on learning and spatial memory using EB-induced demyelination. One of the possible tools to investigate insights of the possible link between vitamin D₃ and NO in complex procedure of cognition is NO concentration measurement. NO and peroxynitrite (ONOO−) are considered of reactive nitrogen species (RNS). Increased production of RNS and reactive oxygen species (ROS) in pathological circumstances changes the oxidant and antioxidant balances and harmfully causes several pathological diseases.

Vitamin D₃ influences neurodegenerative diseases through multiple mechanisms. This vitamin plays potential roles in a number of physiological processes and may provide neuroprotection through preservation from cytotoxicity and also by keeping the balance of antioxidative pathway. Today, experimental and preclinical data suggest a link between vitamin D₃ status and cognitive function (Landel et al., 2016).

According to the above information, this study was designed and performed to clarify the therapeutic effect of vitamin D₃ on the cognitive disorders and its mechanism of action in context of demyelination model.

**MATERIAL AND METHODS**

**Animal training**

Male Wistar rats weighing 200-250 g (8-10 weeks old) were purchased from Pasteur institute, Tehran, Iran. Before surgery every five rats were kept in one cage. They had free access to food and water and kept on 12 hours light cycle. Room temperature was adjusted at 25 ± 2 °C. Ethical committee of the Alborz University of Medical Sciences granted the license of laboratory works on animals according to the regulations.

Rats were anesthetized by intraperitoneal (IP) injection of chloral hydrate (80 mg/kg). Stereotaxic device was used to fix rat’s head in order to locate right dentate gyrus to be injected by EB (Levine, Reynolds, 1999). Rats divided into 4 groups of 10 in each. First group was injected normal saline instead of EB in hippocampus to be used as a healthy control. Other groups were injected EB (3 µl, 0.01% in normal saline, Cinnagen Co.) in the right dentate gyrus but treated differently. Group II was injected EB alone. After surgery, groups III and IV were injected Vitamin D₃ (5µg/kg, DSM Nutritional Products, Village-Neuf, France) IP for 7 (D-7) and 28 days (D-28), respectively. Dose of vitamin D₃ was determined according to some reports and our own former investigation (Garçon et al., 2003; Goudarzvand et al., 2010). As all injections were done unilaterally into a small area of hippocampus, movement disorder was not expected, but animals showing movement disorder were excluded from the study. We used sesame seed oil as the solvent for vitamin D₃ as in our previous study it was shown to be inert for our behavioral study (Goudarzvand et al., 2010).

Behavioral experiments were performed according to five days protocol of the Morris water maze (MWM) procedure (Dringenberg et al., 2001; Naghdí, Oryan, Etemadi, 2003). It was done with hidden platform for 4 days to evaluate learning and spatial memory. Using a visible platform, assessment of sensory motor coordination was done on the fifth day. Platform was in third zone of pool that was called as target zone in the article.
Spatial memory is the process to store any information about the spatial orientation of the live subject. In hidden platform experiments, each rat was given 4 daily trials for 4 days in which a random set of four different start locations (north, south, west, and east) was used. Each time rats were given 60 seconds to reach the platform. If rats could not reach the platform before 60 seconds, they were guided to platform, gently. Rats had 30 seconds time to stay on the platform and evaluate the surrounding. On the fifth day platform was covered with aluminum foil and located 1 cm above water. Reaching the platform by rats was related to their motor and visionary health. After every day experiment animals were dried and transferred to home cages.

Travelled distance, escape latency (time spent to reach the platform) and time spent in target zone were used to evaluate spatial learning and memory. Swimming speed was considered as an index of sensory motor system’s functionality. Data represented as mean of 10 experiments ± standard deviation. These experiments were recorded.

**NO concentration assay**

Nitric oxide exposed to oxygen is rapidly oxidized to nitrite ion (NO$_2^-$). Therefore, nitrite level was measured to show nitric oxide changes in the hippocampus. After the last memory experiment, rats were decapitated and hippocampus was dissected out of the brain. The hippocampus tissue was homogenized in 3-ml ice-cold phosphate buffered saline (0.1 M, pH: 7.4). After centrifugation at 12000 g at 4°C, supernatant was used for nitrite level determination according to Griess reaction assay (Green et al., 1982) in which nitrite compounds react with reagents (0.1% N-(1-naphtyl) ethylenediaminedihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) to appear a purple azo color. Absorbance is measured by a spectrophotometer at 540 nm. Standard curve obtained from different concentrations of sodium nitrite. Protein level of each sample was determined by Lowry method (Lowry et al., 1951). Nitrite ion concentration was calculated as µmol per mg of protein.

**Statistical analysis**

The data were analyzed by SPSS software version 23 and one-way ANOVA and also Tukey post hoc test were used for comparison between the different groups with the control. It was performed at a significance level of p<0.05.

**RESULTS AND DISCUSSION**

Injection of ethidium bromide, as a local demyelination model, in the hippocampus caused a significant increase in the travelled distance (p<0.05) and the time spent to reach the platform (p<0.05) compared with the saline group (Figure 1A and Figure 1B). Administration of vitamin D$_3$ for 7 days post lesion did not cause any significant difference compared to EB group, however, its administration for 28 days, after injury, resulted in a significant decrease in the travelled distance and the escape latency (time spent to reach the platform) in comparison to EB group for both (p<0.05; Figure 1A and Figure 1B). Figure 1C reflected the swimming rate as a control to ensure normal sensory-motor coordination. There was no significant difference among different groups in swimming rate as expected. Otherwise, data obtained were unacceptable and should have been excluded from the study. It should be reminded that swimming rate is an indicative of sensory-motor proper function showing that animals can swim at their own pace.
Results of travelled distance and time spent in different zones of MWM (quadrant 3 was the target zone) indicated that taking vitamin D$_3$ for 28 days (and not for 7 days) led to a significant increase in the travelled distance and time spent in target zone, comparing to other zones (p<0.05; Figure 2A and Figure 2B).
Nitric oxide (NO) concentration was measured after the last behavioral study in different groups. As shown in Figure 3 injection of ethidium bromide in hippocampus caused a significant increase in NO concentration (µmol per mg of protein) in comparison with saline group (p<0.05) whereas NO concentration in groups receiving vitamin D₃ for 28 days had significant decrease compared to EB-28 group (p<0.05). Administration of vitamin D₃ for 7 days could not develop a significant decline in NO amount, however (Figure 3).
In this study demyelination was induced by injection of EB into hippocampus area of young rats (Blakemore, Crang, Evans, 1983; Mazzanti et al., 2009; Sailer et al., 2003). According to cognitive tests, EB group showed obvious detrimental influences compared to saline (control) group as a result of induced apoptosis and subsequent demyelination (Blakemore, 1982; Blakemore, Crang, Evans, 1983; Goudarzvand et al., 2010).

There are a large number of investigations that have confirmed the positive effects of vitamin D3 on cognitive functions (Erbaş et al., 2014; Goudarzvand et al., 2010; Mosayebi, Ghazavi, Payani, 2006; Van der Schaft et al., 2013). Studies featuring the vitamin D3 effect on cognition can be interpreted through different types. One explains the relation between vitamin D3 deficiency and cognitive dysfunctions (Keeney, Butterfield, 2015; McCann, Ames, 2008). By now most of studies have supported the association of vitamin D3 with neurodegenerative diseases like Alzheimer disease. The other type of vitamin D articles is about the positive preventive effects of vitamin D3 on brain function. The Prophylactic neuroprotective effect of vitamin D is also well documented (Keeney, Butterfield, 2015; Taghizadeh, Talaei, Salami, 2013). However, effect of vitamin D3 supplement on the improvement of cognitive decline, post disorder, has not been fully cleared (Pettersen, Fontes, Duke, 2014). The main finding of our study was that intake of vitamin D3 for 28 days, post injury, made a distinguished improvement in behavioral performance as assessed by MWM procedure. This issue is in agreement with a number of reports Mosayyebi and colleagues (2006), Erbas and colleagues (2014) and Goudarzvand and colleagues (2010). The reason for this positive effect may rely on the antioxidative effects of vitamin D3 that diminishes oligodendrocyte cell death. Opposite results are also reported. One example is a study conducted by Taghizadeh and colleagues (2013). In which vitamin D3 deficiency decreased the spatial learning capacity; however, its supplement did not improve memory performance as shown by behavioral tests. Our results supported memory-improving effects of the vitamin D3 as a potential treatment and suggested that vitamin D3 may be used not only as a pretreatment but also as a post injury treatment for cognitive disorders.

Vitamin D-7 group did not show any significant cognitive progress in MWM behavioral test because the remyelination takes a few weeks to accomplish (Goudarzvand et al., 2016; Goudarzvand et al., 2010).

We also found that NO concentration in D-28 group was changed, dramatically. EB group showed
large increase in NO concentration in addition to significant cognitive impairment compared to the control group. This is in agreement with some researches as a consequence of severe demyelination and apoptosis (Abdel-Salam, Khadrawy, Mohammed, 2012; Garthwaite et al., 2002; Leon-Chavez et al., 2006). The result of MWM test in D-28 group is supported by NO measurement test as cognitive improvements are accompanied by significant decrease in concentration of NO in the hippocampus.

CONCLUSION

It is concluded that vitamin D₃ inhibits induced nitric oxide synthase (iNOS) (Dulla et al., 2016; Dursun, Gezen-Ak, Yilmazer, 2013). Our data confirms the Involvement of vitamin D₃ signaling through NO pathway in the brain that is in agreement with a number of studies (Austin, Santhanam, Katusic, 2010; Chu, Heistad, 2010; Eyles et al., 2003; Garcion et al., 2003). However, there are some controversial records about the effect of vitamin D₃ on NO signaling. This study suggests that NO pathway might have a destructive effect on cognition and spatial memory (Beckmann et al., 2014; Limón et al., 2009; Udayabanu et al., 2008; Wiley, Willmore, 2000; Yu et al., 2013), however, further studies are still needed to clear the exact mechanisms involved, as contradictory results are also published (Martínez-González et al., 2014; Rockett et al., 1998).

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