Cell Mol Neurobiol (2010) 30:289–299
DOI 10.1007/s10571-009-9451-x

ORIGINAL RESEARCH

Vitamins E and D3 Attenuate Demyelination and Potentiate Remyelination Processes of Hippocampal Formation of Rats Following Local Injection of Ethidium Bromide

Mahdi Goudarzvand · Mohammad Javan · Javad Mirnajafi-Zadeh · Sabah Mozafari · Taki Tiraihi

Received: 6 May 2009 / Accepted: 26 August 2009 / Published online: 19 September 2009

Abstract Cognitive deficits have been observed in patients with multiple sclerosis (MS) due to hippocampal insults. Antioxidant vitamins D and E are suggested for patients suffering from neurodegenerative diseases like MS, while their mechanisms of action are not well understood. Here, we have tried to study the effects of these vitamins on demyelination, cell death, and remyelination of rat hippocampus following local ethidium bromide (EB) injection. Animals received 100 mg/kg vitamin E or 5 μg/kg of vitamin D3 for 2, 7, or 28 days. The extent of demyelination, myelin staining intensity, and expression of myelin basic protein and caspase-3 were investigated using histological and immunoblotting verification. Administration of EB alone caused demyelination, cell death, and afterward an endogenous repair. Vitamins E and D3 reduced the EB-induced damage and increased the endogenous remyelination of hippocampus. Although the anti-apoptotic effect of these vitamins and protection against demyelination were predictable based on their antioxidant effect, our results indicated the positive effect of vitamins E and D3 on process of remyelination by endogenous progenitor cells and supported their possible therapeutic effects in the context of demyelinating diseases like MS.

M. Goudarzvand · M. Javan (✉) · J. Mirnajafi-Zadeh · S. Mozafari
Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
e-mail: mjavan@modares.ac.ir

T. Tiraihi
Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Keywords Demyelination · Remyelination · Ethidium bromide · Cell death · Myelin basic protein · Vitamin E · Vitamin D3 · Hippocampus · Endogenous progenitor cells · Rat

Abbreviations

CNS	Central nervous system
EB	Ethidium bromide
MS	Multiple sclerosis
MBP	Myelin basic protein
OPCs	Oligodendrocyte precursor cells
SVZ	Sub-ventricular zone
EAE	Experimental autoimmune encephalomyelitis
DG	Dentate gyrus

Introduction

Regeneration in adult mammalian central nervous system (CNS) fails for different reasons including the presence of inhibitory molecules associated with CNS macroglia and low regenerative potential of most damaged CNS axons. Nevertheless, CNS is capable of regeneration given an appropriate environment or manipulations that restore the regenerative potential of neurons (Brecknell and Fawcett 1996). Demyelination is a pathological event in CNS in that it may be followed by remyelination, a spontaneous regenerative response in which lost myelin internodes are replaced by new ones, resulting in the restoration of saltatory conduction and demyelination-associated loss of function (Smith et al. 1979; Woodruff and Franklin 1999). Recent research, especially using MRI technique, has indicated that demyelination takes place in gray matter, in

addition to its occurrence in white matter (Sailer et al. 2003). Making use of MRI, demyelination lesions, and abnormalities in hippocampus have been reported in patients with neurodegenerative diseases (Vercellino et al. 2005; Roosendaal et al. 2008; Geurts et al. 2007).

Experimentally induced demyelination through chemical agents provides a model system for analyzing the cellular changes that occur during demyelinating pathologies such as MS (Blakemore and Crang 1989; Levine and Reynolds 1999). Direct injection of ethidium bromide (EB) as a chromatin-disrupting agent has offered a simple, reproducible, and powerful model for studying the biology of remyelination and the subsequent repair process (Blakemore 1982; Woodruff and Franklin 1998). Oligodendroglial damage induced by EB produces cell death and primary demyelinating lesions at the site of injection (Blakemore et al. 1983; Bondan et al. 2000, 2006; Guazzo 2005). Reactive T cells and macrophages in CNS produce cytokines and other mediators like tumor necrosis factor α, interferon γ, and reactive oxygen species (H_2O_2, O_2^-, NO) that activate caspase protein family (Rus et al. 2006). There are 14 members in caspase family of proteins which, according to their function, are divided to initiator caspases (caspases 2, 8, 9, and 10), executioner caspases (caspases 3, 6, and 7), and caspases involved in cytokine processing (caspases 1, 4, 5, 11, 12, 13, and 14) (Wolf and Green 1999). Apoptosis contributes to oligodendrocyte depletion in MS lesions and ultimately increases demyelination (reviewed in Rus et al. 2006; Frohman et al. 2006). Also, oligodendrocytes of CNS are very sensitive to oxidative stress in vitro, apparently due to their low capacity for antioxidant defense and intrinsic risk factors (Almazan et al. 2004). Activated immune cells also affect the expression of myelin basic proteins (MBPs) by secreting cytokines (Hung et al. 2002).

Myelin basic proteins are a family of positively charged proteins that contribute to formation and compaction of myelin sheath. In the CNS, such proteins are found in myelinating oligodendrocytes at the major dense line, the cytoplasmic interface of the myelin sheath (Monuki and Lemke 1995). During myelination, the mentioned proteins are expressed in a highly coordinated sequence (Woodruff and Franklin 1998). Of the four major isoforms of MBPs, the proportion of the 21.5 and 17 kDa isoforms is enriched during active myelination (Barbarese et al. 1978).

Antioxidant α-tocopherol is well known as ROS scavengers in vivo and in vitro. The antioxidant activity of α-tocopherol in preventing free radical-initiated peroxidative tissue damage is accepted by most investigators and is believed to be the primary free radical scavenger in mammalian cell membrane (Rose and Bode 1993; Droge 2002). The direct action of α-tocopherol has been attributed to its ability in functioning as a lipid-based free radical chain-breaking molecule, which protects the organism against these radicals (Miyoshi et al. 2005; Traber and Atkinson 2007) and, thereby, prevents from apoptosis and necrosis (Ikeda et al. 2003; Kang et al. 2006).

During the 1970s, the concept that vitamin D might represent an important factor in MS emerged from epidemiological and genetic studies (Goldberg 1974a, b). 1,25-Dihydroxyvitamin-D3 (1,25-D3), the biologically active form of vitamin D, ameliorated the symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model of MS when administered after the onset of clinical signs (Garcion et al. 2003; Mosayebi et al. 2006). Although antioxidant vitamins like vitamin D are recommended as supplements for different therapeutic drugs used for controlling the symptoms of MS, the mechanism of their positive effect is not yet understood. It is not clear that these vitamins are protective, because of their known antioxidant effect and ameliorate the degenerative processes and/or other mechanisms like potentiating the regenerative capacities of CNS are also involved. In this study, we have tried to investigate the effects of vitamins E and D3 on degeneration and remyelination processes in insults induced by local injection of the toxin ethidium bromide.

The adult hippocampus, a vital center for learning and memory, is extremely vulnerable to various insults and neurological diseases (Nakafuku et al. 2002). Cognitive deficits have been observed in 25–60% of MS patients suffering from hippocampus insults (Rao et al. 1991). This area is a site for functionally significant oxidative damage (Cha et al. 2002). Dentate gyrus (DG) of hippocampus is the most mitotically active zone in adult CNS, where granule cells continue to be generated throughout the life in rodents and humans (Becq et al. 2005). The presence of both degenerative and regenerative mechanisms in hippocampus makes it suitable for investigating our questions. Here, we also report the presence of the endogenous remyelinating mechanism in rat's hippocampus in the context of EB-induced demyelination.

Experimental Procedures

Animals

All experiments were carried out on adult male Sprague-Dawley rats (8–10 weeks aged) weighting 200–250 g (Razi Institute, Karaj, Iran). Animals were housed four per cage under a 12-h light/dark cycle in a room with controlled temperature (23 ± 2°C). Food and water were available ad libitum. All research and animal care procedures were performed according to international guidelines on the use of laboratory animals and were approved by Tarbiat Modares University ethical committee for animal

research. Efforts were made to minimize the number of animals used and their suffering.

Gliotoxin Injection and Treatments

Animals were anaesthetized with chloral hydrate (80 mg/kg, i.p.) and placed on rat stereotaxic instrument in the skull-flat position. Demyelination was induced unilaterally by direct single injection of 3 µl of 0.01% ethidium bromide in sterile 0.9% saline (Sim et al. 2000) at the rate of 1 µl/min into the right dentate gyrus of hippocampal formation, using appropriate stereotaxic coordinates (AP = −2.8; ML = +1.8; DV = +2.5) (Paxinos and Watson 2006). Animals in experimental groups received 100 mg/kg vitamin E (DSM Nutritional Products, Village-Neuf, France) (Martinovits et al. 1986; Offen et al. 2001; Cinthia et al. 2009) or 5 µg/kg vitamin D3 (DSM Nutritional Products, Village-Neuf, France) (Garcion et al. 2003) for 2, 7, or 28 days post lesion. Soybean oil (Hayes et al. 2004) as the vehicle of vitamins E and D3 was used as control.

Histological Assessment

Animals were re-anesthetized on days 2, 7, or 28 post lesion and perfused intracardially with freshly prepared phosphate-buffered saline (PBS) and then with 4% paraformaldehyde prepared in 0.1 M phosphate buffer with pH 7.4. The right hemisphere was removed and then postfixed overnight in the same solution at 4°C. The hemispheres were afterward processed and embedded in paraffin, sagitally sectioned at 5-µm-thick preparations. Preparations corresponding to the area between 0.5 mm before the site of injection to 0.5 mm after the site of injection (200 sections) were considered for evaluation. Sections were stained with luxol fast blue (British Drugs Houses Inc, UK) and cresyl fast violet (Merck, Darmstadt, Germany). One section out of each 10 sections (10 sections for each hippocampus) was considered for measuring the extent of demyelination and the intensity of myelin staining (myelination intensity) using light microscopy. In histological analysis, Scion and Image J softwares were used for assessing the myelination intensity and the extent of demyelination, respectively. For each section, the extent of demyelination was assessed as the percentage of total hippocampal area. Myelination intensity was assessed in reference to an intact area in the same preparation. For each animal, data from different sections were averaged and incorporated into the comparison of different groups.

Immunoblotting Analysis

For Immunoblotting, animals were killed by decapitation under CO_2 anesthesia and the right hippocampus was immediately removed and preserved in liquid nitrogen until the day of immunoblotting. The extracted hippocampus was homogenized on ice in cold lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Triton X-100, 0.25% sodiumdeoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% protease inhibitor cocktail (Roche, Germany). After centrifugation, the supernatant was collected and assayed for protein concentration using the Bradford method. The lysates containing equal amounts of protein were resolved on SDS–12.5% polyacrylamide gel electrophoresis, transferred to PVDF membrane (Amersham Bioscience, Freiburg, Germany), blocked in 2% ECL advanced kit blocking reagent (Amersham Bioscience, Freiburg, Germany) and probed with primary rabbit monoclonal antibody to caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal antibody to MBP (1:1000, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. After washing, membranes were incubated for 60 min at room temperature with the horseradish peroxidase–conjugated secondary antibody (1:10000, Santa Cruz Biotechnology, CA, USA). Blots were subsequently revealed by ECL advanced kit (Amersham Bioscience, Freiburg, Germany). To normalize for protein content of lanes, blots were stripped in stripping buffer containing 100 mM 2-mercaptoethanol, 2% (w/v) SDS, and 62.5 mM Tris–HCl (pH 6.7) and then probed with rabbit anti-actin antibody (1:1000, Santa Cruz Biotechnology, CA, USA).

Statistical Analysis

Following immunoblotting, band densities on the blots were measured using Labworks software (UVP, Inc., UK). The densities obtained for activated caspase-3 and MBP were normalized to corresponding actin band density. Data from immunoblotting and histological analysis were scrutinized using one-way analysis of variance (ANOVA) followed by the Tukey post hoc. The results are expressed as mean ± SEM. $P < 0.05$ was considered as minimum significant difference of means.

Results

EB-induced Apoptosis, Demyelination, and Remyelination

EB induced an obvious demyelination in rat hippocampus. The extent of demyelination and the intensity of myelination were studied 2, 7, 14, 28, and 60 days after EB injection using luxol-fast-blue myelin-specific staining. Two days after injection, infiltration of inflammatory cells was usually observed. There was nevertheless no obvious

demyelinated area and the myelination intensity was same as that of control animals (Fig. 1a, b). Demyelinated area was observed on day 7 and increased on days 14 and 28 ($P < 0.001$). Lower level of demyelination was seen on day 60 post lesion. Myelination intensity decreased on days 7, 14, 28 ($P < 0.001$), and 60 ($P < 0.01$), and increased on day 60 ($P < 0.01$ vs. day 28), while it was still below the control level (Fig. 1a, b). Representative histological micrographs for control group and days 7, 28, and 60 are shown in Fig. 1c.

To study the possible role of apoptotic cell death in the process of demyelination and changes in the activity of myelinating cells, we evaluated the expression of caspase-3 and MBP on days 2, 14, and 28 post lesion. Our results showed that there was no significant difference between intact and saline-treated animals for expression of caspase-3 and MBP. Therefore, the data for these different groups were averaged and presented as control. EB injection caused a significant increase in the level of activated caspase-3 on 2, 7, and 28 days post lesion ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively). This increase was at maximum on day 7 (Fig. 2a). EB injection also decreased the expression of MBP on day 2 post lesion ($P < 0.001$) and increased its expression on day 28 ($P < 0.05$). Compared to day 2, the expression of MBP increased on days 7 and 28 (both, $P < 0.001$) (Fig. 2b). Representative western blotting bands are presented in Fig. 2c. Our overall observation showed that the total number of cells did not change in post-lesion days.

Effect of Vitamins E and D3 on EB-induced Demyelination

As is shown in Figs. 3 and 4, a very low demyelination and normal myelination intensity were observed on day 2 post lesion (Figs. 3a, 4a). One-way ANOVA showed a non-significant difference between the groups on day 2, therefore no post-test was carried out. Seven days after EB injection, demyelination was significantly extended and the intensity of myelin staining reduced. However,

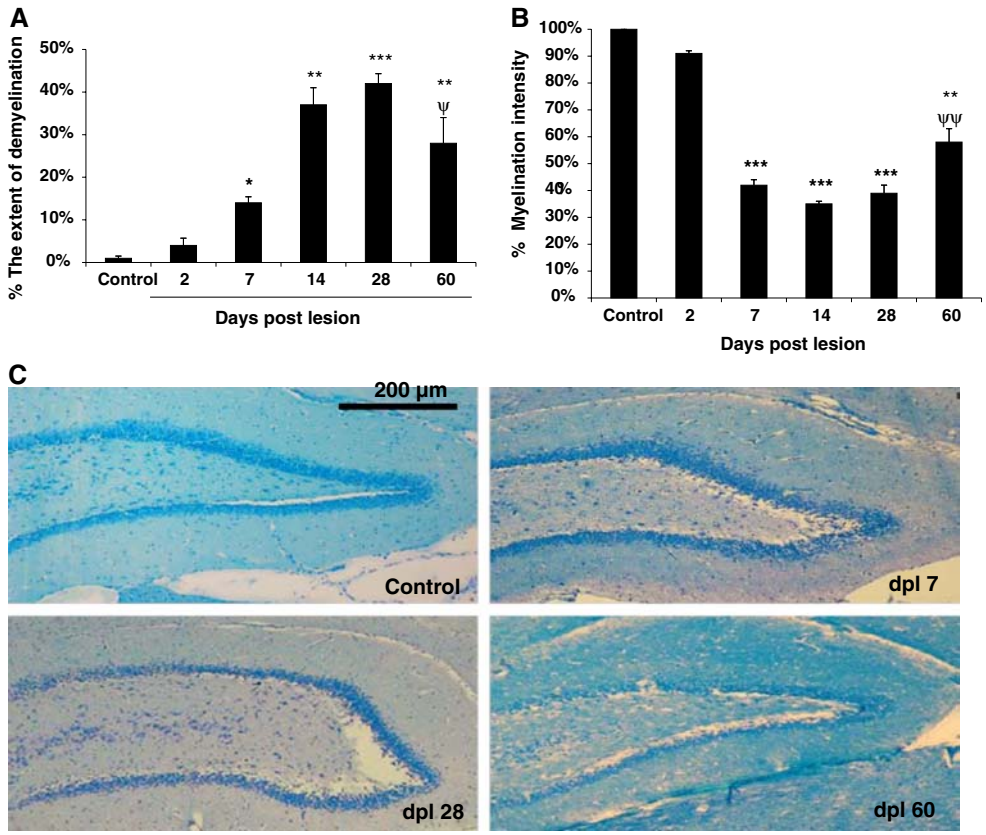

Fig. 1 Ethidium bromide (EB)-induced demyelination and remyelination in rat hippocampus. The extent of demyelination and the intensity of myelin-specific staining were studied 2, 7, 14, 28, and 60 days after local injection of EB, using luxol fast blue staining of histological preparations. **a** A significant demyelinated area was observed on days 7, 14, 28, and 60 post lesion. Lower extent of demyelination was observed on day 60. **b** Myelin-specific staining showed a reduced myelination on days 7, 14, 28, and 60. The myelination level was partially reversed on day 60. **c** Light micrographs for control and days 7, 28, and 60 are shown as representative. *Bars* mean + SEM, $n = 4$, dpl = day post lesion, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control group, † $P < 0.05$, †† $P < 0.01$ compared to day 28 as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

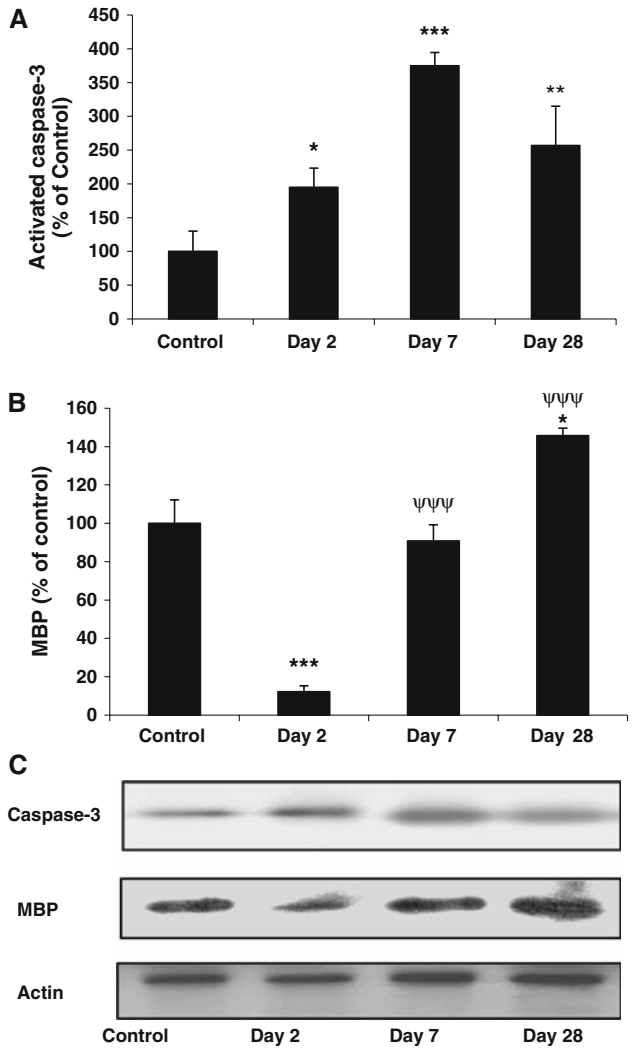

Fig. 2 Changes in the level of activated caspase-3 and MBP proteins following ethidium bromide (EB)-induced demyelination in rat hippocampus, as evaluated by western blotting. Extracts from rat hippocampus were probed with anti-activated-caspase-3 or anti-MBP and then with HRP-conjugated secondary antibody. ECL advanced kit was used to reveal the bands. **a** EB injection caused a significant increase in the level of activated caspase-3 on days 2, 7, and 28 post lesion. This increase was at maximum on day 7. **b** EB injection decreased the expression of MBP on day 2 post lesion. While the expression was at minimum on day 2, it was reversed to control level on day 7 and increased to a higher level on day 28. **c** This is representative of western blot data for caspase-3 and MBP proteins. *Bars* mean + SEM, Control = a pool of intact and saline-treated animals, $n \geq 4$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control group, ††† $P < 0.001$ compared to day 2 post lesion as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

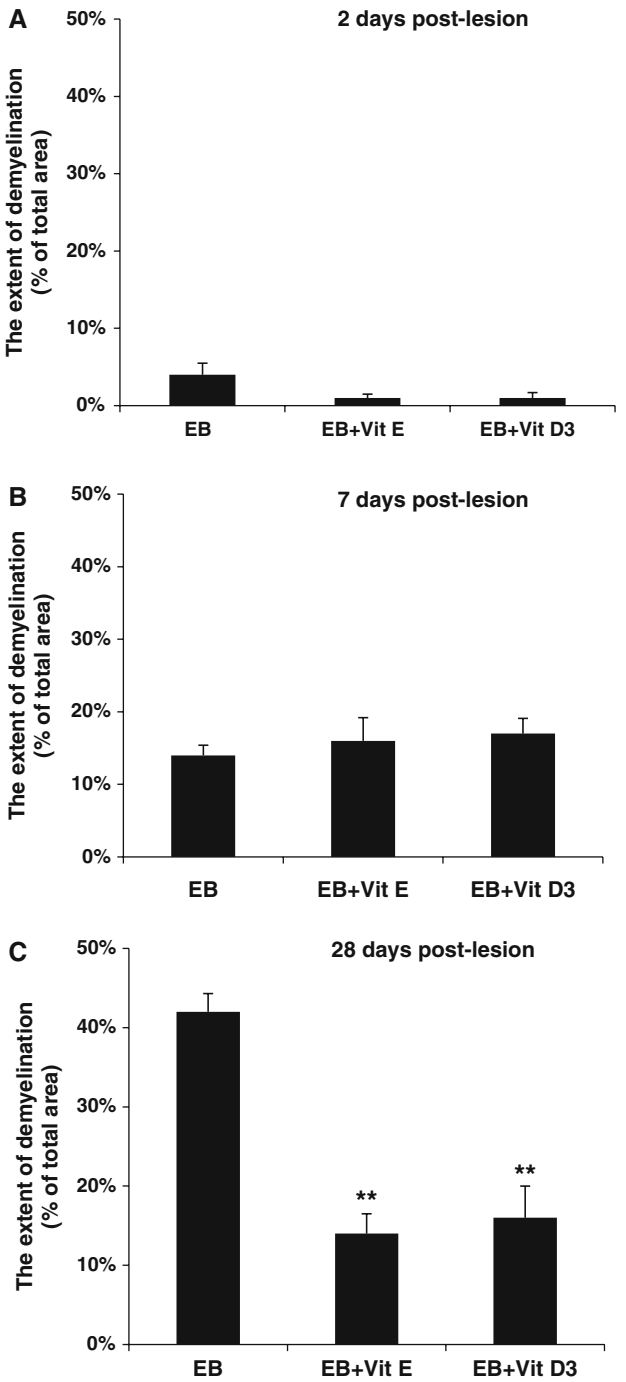

Fig. 3 Effects of vitamins E and D3 on the extent of demyelination induced by injection of ethidium bromide (EB) into rat hippocampus in days 2 (**a**), 7 (**b**), and 28 (**c**) post lesion. Administration of vitamins E and D3 for 2 or 7 days did not exert significant effect on the extent of demyelination. Administration of vitamins E and D3 during 28 days post lesion significantly reduced the extent of demyelination. *Bars* mean + SEM, $n \geq 4$, ** $P < 0.01$ compared to EB-treated group as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

administration of vitamins E or D3 during these 7 days did not show any significant effect on demyelination and myelin staining intensity (Figs. 3b, 4b).

Twenty-eight days after EB injection, animals which received vitamins E and D3 daily showed a significant decrease in the extent of demyelination (both $P < 0.01$) and had an increased level of myelin staining ($P < 0.001$ and $P < 0.05$, respectively) (Figs. 3c, 4c). Our overall

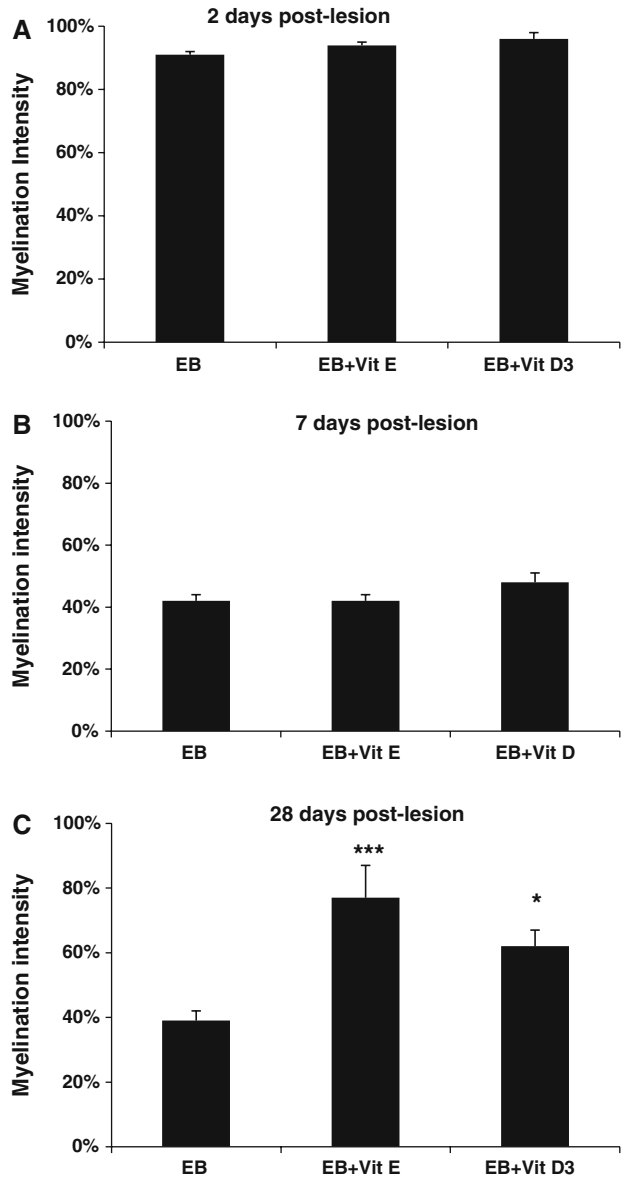

Fig. 4 Effects of vitamins E and D3 on the intensity of myelination following ethidium bromide (EB)-induced demyelination in rat hippocampus in days 2 (**a**), 7 (**b**), and 28 (**c**) post lesion. Administration of vitamins E and D3 for 2 or 7 days did not exert significant effect on the intensity of demyelination. Administration of vitamins E and D3 during 28 days post lesion significantly increased the intensity of myelination. *Bars* mean + SEM, $n \geq 4$, * $P < 0.05$ and *** $P < 0.001$ compared to EB-treated group as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

observation showed that the total number of cells did not change in animals treated with vitamins D3 and E.

In the presence of vitamins E and D3, the extent of demyelination and myelination intensity on day 28 seems to be same to, or even better than these parameters on day 60 in control group. On the other word, vitamins shortened the recovery time to about 50%. Based on this observation, we ignored day 60 from vitamin-treated groups.

Effect of Vitamins E and D3 on EB-induced Apoptosis and Remyelination

Our data showed that EB injection into hippocampus induces apoptosis, demyelination, and remyelination. To study the effects of daily administration of vitamins E and D3 on the level of activated caspase-3, demyelination and remyelination, the levels of activated caspase-3 and MBP were measured 2, 7, and 28 days after EB injection. Since there was no difference in the level of caspase-3 and MBP in intact and vehicle-treated animals, the data for these two groups were merged and mentioned as control.

As is shown in Fig. 5, 2 days after the injection of EB, the level of activated caspase-3 ($P < 0.05$) increased significantly. This increase was reversed by vitamins E and D3 (Fig. 5a). Seven days after EB injection, the level of activated caspase-3 increased highly. Vitamins E and D3 returned the activated caspase-3 level to control level (both $P < 0.001$ vs. EB-treated rats) (Fig. 5b). The level of activated caspase-3 in EB-treated rats on day 28 post lesion was still higher than that of control. This level was significantly decreased by vitamins E and D3 ($P < 0.001$ and $P < 0.01$, respectively). Vitamin E decreased the level of activated caspase-3 to a lower level than control ($P < 0.01$) (Fig. 5c).

As is shown in Fig. 6, while EB alone severely decreased the expression of MBP on day 2 post lesion, vitamins E and D3 reversed this effect (both $P < 0.05$). The expression of MBP in vitamin E-treated animals was significantly higher than that in EB-treated group ($P < 0.05$) (Fig. 6a). On day 7, there was no significant difference among control, EB-, vitamin E-, and vitamin D3-treated animals (Fig. 6b). On day 28, the expression level of MBP in EB-, vitamin E- and vitamin D3-treated animals was higher than that in control group ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). Compared to the EB-treated group, administration of vitamins E and D3 significantly increased the expression of MBP (both $P < 0.001$) (Fig. 6c).

Discussion

Several studies have proposed that demyelinating insults occur in the CNS gray matter of MS patients. Hippocampal formation is known as one of the important gray matters which are reported to be affected by MS (Sailer et al. 2003; Geurts et al. 2007). The purpose of the present study was to investigate the effect of vitamins D3 and E on the cell death and remyelination of rat hippocampal formation following local injection of EB. EB injection into hippocampus caused local demyelination. Maximum extent of demyelination was observed on day 28 post lesion. Sixty

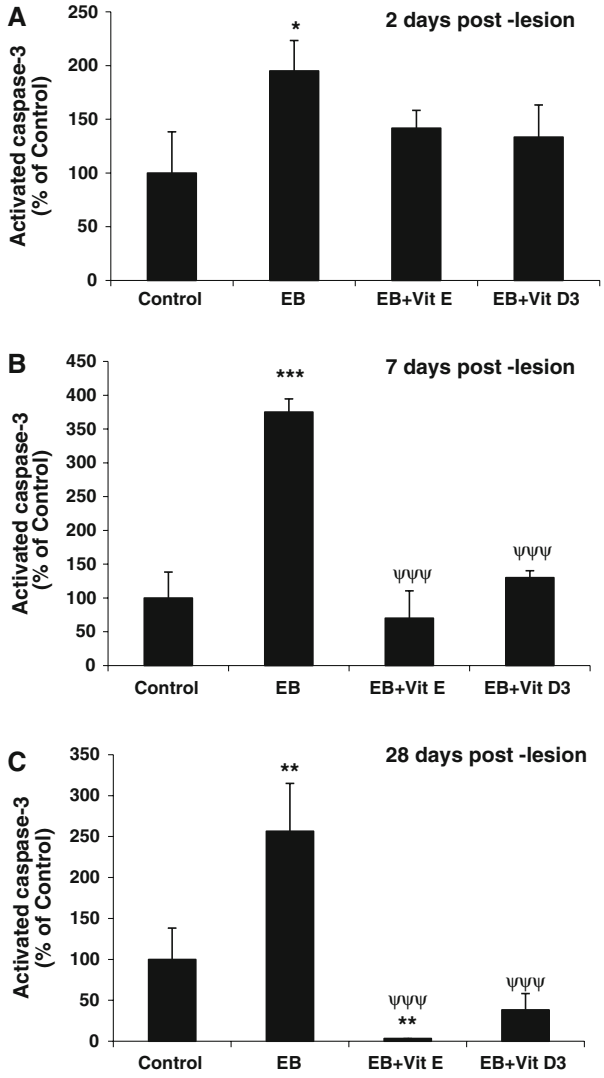

Fig. 5 Effects of vitamins E and D3 on the ethidium bromide (EB)-induced changes in the level of activated caspase-3 in rat hippocampus evaluated by western blotting. Extracts from rat hippocampus were probed with anti-activated caspase-3 or anti-MBP and then with HRP-conjugated secondary antibody. ECL advanced kit was used to reveal the bands. **a** This graph shows that vitamins E and D3 prevented the significant increase in the level of active caspase-3 on day 2 post lesion. **b** The graph indicates that administration of vitamins E and D3 during 7 days post lesion significantly decreased EB-induced elevated level of active caspase-3. **c** This graph shows that administration of vitamins E and D3 during 28 days post lesion significantly decreased EB-induced elevated level of active caspase-3. Vitamin E reduced the level of caspase-3 even to a level lower than that of control. *Bars* mean + SEM, Control = a pool of intact and saline-treated animals, $n \geq 4$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control group, †† $P < 0.01$, ††† $P < 0.001$ compared to EB-treated animals as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

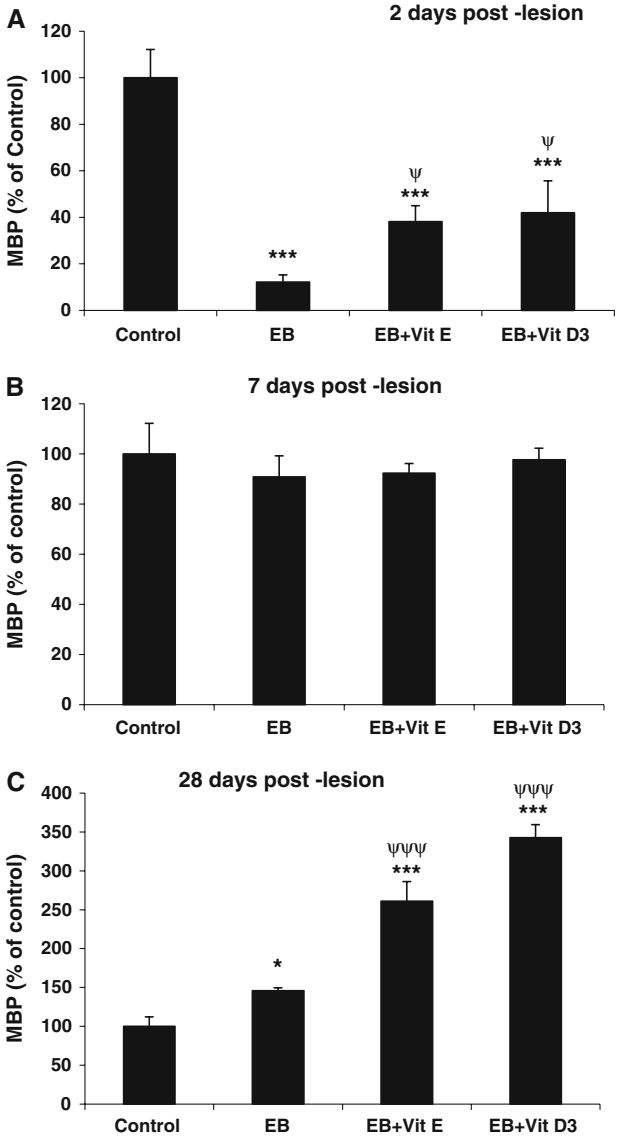

Fig. 6 Effects of vitamins E and D3 on the ethidium bromide (EB)-induced changes in the level of MBP in rat hippocampus evaluated by western blotting. Extracts from rat hippocampus were probed with anti-activated caspase-3 or anti-MBP and then with HRP-conjugated secondary antibody. ECL advanced kit was used to reveal the bands. **a** On day 2 post lesion, the expression of MBP in animals treated with vitamin E was significantly higher than EB-treated group. **b** On day 7 post lesion, there was no significant difference between control and EB-, vitamin E-, or vitamin D3-treated animals. **c** On day 28 post lesion, the expression level of MBP in EB-, vitamin E-, and vitamin D3-treated animals was higher than that in control group. Compared to EB-treated group, administration of vitamins E and D3 significantly increased the expression of MBP. *Bars* mean + SEM, Control = a pool of intact and saline-treated animals, $n \geq 4$, * $P < 0.05$, *** $P < 0.001$ compared to control group, † $P < 0.05$, ††† $P < 0.001$ compared to EB-treated animals as evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc

days post lesion, the extent of demyelination area reduced and a higher myelin-specific staining was observed. The presence of both demyelinating and remyelinating processes following local injection of EB into hippocampus provides a well-suited model for studying the effect of potentially effective drugs on demyelinating diseases like MS. The endogenous remyelination in this experiment also

provides a good model for investigating the endogenous mechanisms of CNS repair. Our results also showed that subsequent to local injection of EB, an increased level of the apoptotic protein, activated caspase-3, was observed on days 7 and 28. This finding was similar to previous reported results on apoptosis caused by EB administration in CNS. This apoptosis may be due to activation of T-cells and macrophages which produce inflammatory cytokines and mediators such as tumor necrosis factor [TNF]-α, interferon [IFN]-γ and reactive oxygen/nitrogen species (Rus et al. 2006; Frohman et al. 2006). Biochemical events initiated by these reactive species may induce lipid peroxidation and protein oxidation, leading to cell damage and death by apoptosis (Kochman et al. 2002). Degenerative changes, induced by EB injection into rat brain, are reported in oligodendrocytes within 72 h and their subsequent death results in demyelination (Blakemore et al. 1983). Due to their lower antioxidant capacity, oligodendrocytes are more sensitive to oxidative and nitrative stress, compared to microglial cells and astrocytes (Smith et al. 1999). EB causes death in oligodendrocytes via producing free radicals, but this neural damage may be compensated by physiological capacity of CNS for repair (Setzu et al. 2004; Zhang et al. 1999; Levine et al. 2001). Such apoptosis may explain the EB-induced demyelination in our study.

MBP is expressed in myelinating cells including both immature and mature oligodendrocytes and, in this way, its expression can regard as activity or presence of these cells (Baumann and Pham-Dinh 2001). The expression of MBP in the lesioned hippocampus was significantly reduced 2 days post lesion, returned to control level on day 7, and increased to a level higher than that in control on day 28. The level of MBP expression on days 7 and 28 was significantly higher than its expression level on day 2. Reduced level of MBP on day 2 seems to be attributable to oligodendrocytes damage and death as it was assumed by approximately twofold increase in the level of activated caspase-3. The other possibility for decreased MBP expression is the role of activated immune cells. Activated immune cells decrease the expression level of myelin basic proteins by secreting the cytokines (Hung et al. 2002). Following the lesion and death of oligodendrocytes, it is shown that precursor cells migrate into the lesion site and then differentiate to oligodendrocytes and increase the level of MBP expression (Zhao et al. 2005; Sim et al. 2000). Results of our study also confirmed the elevation of MBP expression to control level on day 7. On day 28, the expression level of MBP was even higher than that of control. There are two possible explanations for the higher levels of MBP expression in the remyelinated area. First, it is known that cell proliferation is required for remyelination and that the remyelinated area contains more myelinating oligodendrocytes (Keirstead et al. 1998; Keirstead and Blakemore 1997; Prayoonwiwat and Rodriguez 1993). If oligodendrocytes were expressing similar amounts of MBP then increased number of cells would accounts for the higher levels of myelin protein expression. The second possibility is that the levels of expression reflect the age of the myelinating cell, thus in the remyelinated area being much younger than that in the control white matter (Sim et al. 2000). Our overall observation of the histological preparations showed that the total number of cells did not change in post-lesion days. Therefore, the first explanation seems to be more plausible. Another explanation is that, we have estimated the total number of cells, but the occurrence of apoptosis is more possible for oligodendrocytes, due to their higher sensitivity. In the context of demyelination, progenitor cells also mostly differentiate into oligodendrocytes. Therefore, the other possibility is that in our experiment, the significant change in a small portion of cells is masked by total cell count. In a consistent report, in caspase-3-deficient mouse with a decreased apoptosis and marked expansion of the total population of actively proliferating neuroblasts, the histological features of normal neurogenesis seemed to be maintained (Pompeiano et al. 2000). Totally, our findings in molecular level shows that in post-lesion time, the condition has changed in favor of remyelination, which may lead to increased myelination intensity, as was observed in 60 days post lesion in histological studies.

The endogenous potential of CNS for remyelination of the lesions was previously reported (Zhang et al. 1999; Zhao et al. 2005; Scolding 2001; Rice and Scolding 2007). There may be several sources for myelinating cells in adults CNS; Oligodendrocyte precursors that have widespread distribution throughout the white and gray matters in healthy brain are composed of approximately quiescence populations that proliferate slowly and participate in oligodendrocyte turnover (Levine et al. 2001). It is shown that these cells are able to be activated in response to a demyelinating insult and participated in adults CNS remyelination (Franklin et al. 1997). Subventricular zone of lateral ventricles is the main pool of stem cells in the brain (Galli et al. 2003), which is reported that are able to answer to the demyelinating insults in corpus callosum by proliferation, mobilization to the site of injury, and differentiation into the myelinating cells (Gensert and Goldman 1997; Nait-Oumesmar et al. 2008). The stem cells are also present in hippocampus (Nakafuku et al. 2002; Becq et al. 2005). Toxin-induced lesion in the CNS usually causes inactivation or death of progenitors inside the lesion. Therefore, the repair is thought to be mostly due to migration of myelinating cells from tissues around the lesion (Franklin et al. 1997; Menn et al. 2006).

Based on the epidemiological, genetic, and animal studies, vitamin D might represent an important factor in MS (Goldberg 1974a, b). Although the antioxidant vitamin D is recommended as a supplement for different therapeutic regimes of MS (Garcion et al. 2003), the mechanism of its positive effect is not yet understood. Vitamin E is an essential nutrient in humans and its antioxidant and anti-inflammatory role is well established (Johnson 2000; Rimbach et al. 2002; El-Demerdash 2004; Grammas et al. 2004; Reiter et al. 2007). Vitamin E-treated rats have shown smaller lesions induced by EB, suggesting that this compound somehow interferes in the development of lesions. As is shown in Figs. 3 and 4, administration of vitamins D3 and E for 7 days did not change the extent of demyelination and the intensity of myelin staining. Twenty-eight days administration of vitamins D3 and E significantly reduced the extent of EB-induced demyelination in hippocampus. These vitamins also increased the myelin-specific staining as an index of remyelination. This positive action of vitamins D3 and E may be caused by their protective and anti-inflammatory effects against the oligodendrocytes damage. These results are in accordance with other studies that demonstrated the anti-inflammatory effects of vitamins D3 and E on demyelinating models of MS (Garcion et al. 2003; Hayes et al. 1997; Mosayebi et al. 2006; Cinthia et al. 2009). More focusing on the myelin staining intensity parameter reveals that in day 7 post lesion, EB alone but not in the presence of vitamins D3 and E, caused a significant decrease in the intensity of myelin staining, while in day 28 the vitamins caused a significantly increased myelin staining. In the other words, comparing the myelination intensity in days 7 and 28 in vitamin-treated groups shows a significant increase in the myelin staining. These findings show that the effects of vitamins D3 and E at doses applied in this study may be mostly due to their positive effect on the process of remyelination.

In this study, the positive effects of vitamins D3 and E on demyelination and remyelination processes were also verified by the levels of active caspase-3 and MBP expressions. While the level of activated caspase-3 in day 2 post lesion was significantly higher than that in control group, increase in the level of this protein in animals treated with vitamins D3 and E were non-significant. Seven or 28 days post lesion, vitamins D3 and E reduced the level of caspase-3. The level of this protein in day 28 following vitamin E administration was even lower than that in control group. Altogether, changes in the level of caspase-3 in this experiment support the previously reported effect of antioxidant vitamins on apoptosis of CNS cells. In the other words, the protective effect of vitamins D3 and E on the processes of demyelination and remyelination is at least partially mediated by their effect on the EB-induced apoptosis (Ikeda et al. 2003; Kang et al. 2006; Noseworthy et al. 2000). Seven days post lesion, a lower decrease in MBP expression was observed in animals treated with vitamins E and D3. This effect mostly seems to be owing to reduced damage and apoptosis in myelinating cells. Twenty-eight days post lesion, when the expression of MBP in EB-treated group was higher than that in control level, vitamins D3 and E increased the expression of MBP even to higher levels than EB group. This increased level of MBP following its initial decrease seems to be due to an increase in the number of MBP expressing cells or due to an increased expression level of MBP in recruiting cells (Sim et al. 2000). The total number of cells did not change in vitamin-treated animals. Therefore, as heretofore discussed the second possibility seems to be more plausible. In the other word, after 28 days, less caspase-3 is visible in vitamin-treated animals with more myelination. It seems that the newly generated cells which compensate for the apoptosis are more active in producing MBP.

It may be suggested that vitamins D3 and E are capable of increasing the remyelination process through potentiating the remyelination process and decreasing EB-induced apoptosis (Ikeda et al. 2003; Kang et al. 2006; Noseworthy et al. 2000; Hung et al. 2002). Previous studies also suggest the effect of vitamins D3 and E on CNS regeneration and stem cells-mediated regeneration. Many studies have clearly indicated that antioxidant activity of vitamin E protects the neural tissue against a variety of neurodegenerative conditions induced by oxidative stress (Schewe 1995; Imai et al. 2001; Porciuncula et al. 2001; Grammas et al. 2004; Mosayebi et al. 2006; Reiter et al. 2007) and have suggested it as a potential therapeutics and scientific tool to be investigated in brain disorders associated with demyelinating events (Cinthia et al. 2009). Autoradiographic, immunologic, and mRNA expression studies suggest that the neuronal system, including the hippocampal subfields and glial cells, is the potential target of vitamin D (Baas et al. 2000; Clemens et al. 1988). Another study indicated that vitamin D receptor (VDR) protein is present and functional in hippocampus neurons and glial cells (Langub et al. 2001). In vivo data confirm vitamin D-induced increases of NGF levels in rat brain (Saporito et al. 1993, 1994). These reports may explain for the positive effects of vitamin D3 in the context of EB-induced demyelination and remyelination.

Our data showed that vitamins D3 and E were able to protect the CNS against a demyelinating damage. Although this was predictable based on antioxidant and anti-apoptotic effects of these vitamins, their effect on the process of remyelination and increasing the myelination level provided a quite new insight into the potential therapeutic effects of vitamins D3 and E. The positive effect suggested for vitamins D3 and E in the context of demyelinating diseases like MS seems to be a result of their protective

effect on the demyelination process and apoptosis and also their positive effect on proliferation, migration, and differentiation of stem cells into adult myelinating cells.

Acknowledgments This research was supported by grants from Tarbiat Modares University and Iran National Sciences Foundation (grant #87020491).

References

Almazan G, Fragoso G, Martınez-Bermudez AK, Liu HN, Khorchid A, Chemtob S, Mushynski WE (2004) Developmental differences in H2O2-induced oligodendrocyte cell death: role of glutathione, mitogen-activated protein kinases and caspase 3. J Neurochem 90:392–404

Baas D, Prufer K, Ittel ME, Kuchler-Bopp S, Labourdette G, Sarlieve LL, Brachet P (2000) Rat oligodendrocytes express the vitamin D(3) receptor and respond to 1, 25-dihydroxyvitamin D(3). Glia 31:59–68

Barbarese E, Carson JH, Braun PE (1978) Accumulation of the four myelin basic proteins in mouse brain during development. J Neurochem 31:779–782

Baumann N, Pham-Dinh D (2001) Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System. Physiol Rev 81:871–927

Becq H, Jorquera I, Ben-Ari Y, Weiss S, Represa A (2005) Differential properties of dentate gyrus and CA1 neural precursors. J Neurobiol 62:243–261

Blakemore WF (1982) Ethidium bromide induced demyelination in the spinal cord of the cat. Neuropathol App Neurobiol 8:365–375

Blakemore WF, Crang AJ (1989) The relationship between type-1 astrocytes, Schwann cells and oligodendrocytes following transplantation of glial cell cultures into demyelinating lesions in the adult rat spinal cord. J Neurocytol 18:519–528

Blakemore WF, Crang AJ, Evans RJ (1983) The effect of chemical injury on oligodendrocytes. In: Mimms CC, Cuzneks MC, Kelly W (eds) Viruses and demyelinating diseases. Acad Press, London, pp 167–190

Bondan EF, Lallo MA, Sinhorini IL, Pereira LA, Graça DL (2000) The effect of cyclophosphamide on brainstem remyelination following local ethidium bromide injection in Wistar rats. J Submicrosc Cytol Pathol 32:603–612

Bondan EF, Lallo MA, Trigueiro AH, Ribeiro CP, Sinhorini IL, Graça DL (2006) Delayed Schwann cell and oligodendrocyte remyelination after ethidium bromide injection in the brainstem of Wistar rats submitted to streptozotocin diabetogenic treatment. Braz J Med Biol Res 39:637–646

Brecknell JE, Fawcett JW (1996) Axonal regeneration. Biol Rev 71:227–255

Cha CI, Shin CM, Chung YH, Kim MJ, Lee EY, Kim E–G (2002) Age-related changes in the distribution of nitrotyrosine. Brain Res 931:194–199

Cinthia M, Mazzanti CM, Spanevello R, Ahmedb M, Pereira LB, Goncalves JF, Corre M, Schmatz R, Stefanello N, Leal DBR, Mazzanti A, Ramos AT, Martins TB, Danesi CC, Graca DL, Morsch VM, Schetinger MRC (2009) Pre-treatment with ebselen and vitamin E modulate acetylcholinesterase activity: interaction with demyelinating agents. Int J Dev Neurosci 27:73–80

Clemens TL, Garrett KP, Zhou XY, Pike JW, Haussler MR, Dempster DW (1988) Immunocytochemical localization of the 1, 25-dihydroxyvitamin D3 receptor in target cells. Endocrinol 122:1224–1230

Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47–95

El-Demerdash F (2004) Antioxidant effect of vitamin E and selenium on lipid peroxidation, enzyme activities and biochemical parameters in rats exposed to aluminium. J Trace Elem Med Biol 18:113–121

Franklin RJM, Gilson JM, Blakemore WF (1997) Local recruitment of remyelinating cells in the repair of demyelination in the central nervous system. J Neurosci Res 50:337–344

Frohman EM, Racke MK, Raine CS (2006) Multiple sclerosis—The Plaque and Its Pathogenesis. N Engl J Med 354:9

Galli R et al (2003) Neural stem cells an overview. Circ Res 92: 598–608

Garcion E, Sindji L, Nataf S, Brachet P, Darcy F, Montero-Menei CN (2003) Treatment of experimental autoimmune encephalomyelitis in rat by 1, 25-dihydroxyvitamin D3 leads to early effects within the central nervous system. Acta Neuropathol 105: 438–448

Gensert JM, Goldman JE (1997) Endogenous progenitors remyelinate demyelinated axons in the adult CNS. Neuron 19:197–203

Geurts JJ, Bo L, Roosendaal SD, Hazes T, Daniels R, Barkhof F et al (2007) Extensive hippocampal demyelination in multiple sclerosis. J Neuropathol Exp Neurol 66:819–827

Goldberg P (1974a) Multiple sclerosis: vitamin D and calcium as environmental determinants of prevalence. 1. Sunlight, dietary factors and epidemiology. Int J Environ Stud 6:19–27

Goldberg P (1974b) Multiple sclerosis: vitamin D and calcium as environmental determinants of prevalence. 2. Biochemical and genetic factors. Int J Environ Stud 6:121–200

Grammas P, Hamdheydari L, Benaksas EJ, Mou S, Pye QN, Wechter WJ, Floyd RA, Stewart C, Hensley K (2004) Anti-inflammatory effects of tocopherol Metabolites. Biochem Biophys Res Commun 319:1047–1052

Guazzo EP (2005) A technique for producing demyelination of the rat optic nerves. J Clin Neurosci 12:54–58

Hayes CE, Cantorna MT, Deluca HF (1997) Vitamin D and multiple sclerosis. Proc Soc Exp Biol Med 216:21–27

Hayes CE, Spach KM, Pedersen LB, Nashold FE, Kayo T, Yandell BS, Prolla TA (2004) Gene expression analysis suggests that 1, 25-dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by stimulating inflammatory cell apoptosis. Physiol Genom 18:141–151

Hung CJ, Nazarian R, Lee J, Zhao PM, Espinosa-Jeffery A, De Vellis J (2002) Tumor necrosis factor modulates transcription of myelin basic protein gene through nuclear factor kappa B in a human oligodendroglioma cell line. Int J Dev Neuro 20:289–296

Ikeda K, Negishi H, Yamori Y (2003) Antioxidant nutrients and hypoxia/ischemia brain injury in rodents. Toxicology 189:55–61

Imai H, Masayasu H, Dewar D, Graham DI, Macrae IM (2001) Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia. Stroke 32:2149–2154

Johnson S (2000) The possible role of gradual accumulation of copper, cadmium, lead and iron and gradual depletion of zinc, magnesium, selenium, vitamins B2, B6 and E and essential fatty acids in multiple sclerosis. Med Hypoth 55:239–241

Kang SK, Jeong YW, Park SW, Hossein MS, Kim S, Kim JH, Lee SH, Lee BC, Hwang WS (2006) Antiapoptotic and embryotrophic effects of a-tocopherol and L-ascorbic acid on porcine embryos derived from in vitro fertilization and somatic cell nuclear transfer. Theriogenol 66:2104–2112

Keirstead HS, Blakemore WF (1997) Identification of post-mitotic oligodendrocytes incapable of remyelination within the demyelinated adult spinal cord. J Neuropathol Exp Neurol 56: 1191–1201

Keirstead HS, Levine JM, Blakemore WF (1998) Response of the oligodendrocyte progenitor cell population (defined by NG2

labeling) to demyelination of the adult spinal cord. Glia 22: 161–170
Kochman A, Cz Koska, Metodiewa D (2002) Submolecular adventures of brain tyrosine: what are we searching for now? Amino Acid 23:95–101
Langub MC, Herman JP, Malluche HH, Koszewski NJ (2001) Evidence of functional vitamin D receptors in rat hippocampus. Neurosci 104:49–56
Levine JM, Reynolds R (1999) Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp Neurol 160:333–347
Levine JM, Reynolds R, Fawcett JW (2001) The oligodendrocyte precursor cell in health and disease. Trends in Neurosci 24:39–47
Martinovits G, Melamed E, Cohen O, Rosenthal J, Uzzan A (1986) Systemic administration of antioxidants does not protect mice against the dopaminergic neurotoxicity of MPTP. Neurosci Lett 29:192–197
Menn B, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, Alvarez-Buylla A (2006) Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 26:7907
Miyoshi N, Naniwa K, Kumagai T, Uchida K, Osawa T, Nakamura Y (2005) Tocopherol-mediated caspase-3 up-regulation enhances susceptibility to apoptotic stimuli. Biochem Biophys Res Commun 334:466–473
Monuki ES, Lemke G (1995) Molecular biology of myelination, in The Axon. Oxford University Press, New York, pp 144–163
Mosayebi G, Ghazavi A, Payani MA (2006) The effect of vitamin D3 on the inhibition of experimental autoimmune encephalomyelitis in C57BL/6 mice. J Iran Univ Med Sci 13:189–196
Nait-Oumesmar B et al (2008) The role of SVZ-derived neural precursors in demyelinating diseases: From animal models to multiple sclerosis. J Neurol Sci 256:26–31
Nakafuku M, Nakatomi H, Kuriu T, Sh Okabe, Sh-I Yamamoto, Hatano O, Kawahara N, Tamura A, Kirino T (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429–441
Noseworthy JH, Lucchinetti C, Rodriguez M (2000) Multiple sclerosis. N Engl J Med 343:938–951
Offen D, Gilgun-Sherki Y, Melamed E (2001) Oxidative stress induced- neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacol 40:959–975
Paxinos G, Watson C (2006) The rat brain in stereotaxic coordinates, 6th edn. Academic press, New York
Pompeiano M, Blaschke AJ, Flavell RA, Srinivasan A, Chun J (2000) Decreased apoptosis in proliferative and postmitotic regions of the Caspase 3-deficient embryonic central nervous system. J Comp Neurol 423:1–12
Porciuncula LO, Rocha JBT, Boech CR, Vendite D, Souza DO (2001) Ebselen prevents excitotoxicity provoked by glutamate in rat cerebellar granule neurons. Neurosci Lett 299:217–220
Prayoonwiwat N, Rodriguez M (1993) The potential for oligodendrocyte proliferation during demyelinating disease. J Neuropathol Exp Neurol 52:55–63
Rao SM, Leo GL, Bernardin L, Unverzagt F (1991) Cognitive dysfunction in multiple sclerosis. I. Frequency, patterns, and prediction. Neurology 41:685–691
Reiter E, Jiang Q, Christen S (2007) Anti-inflammatory properties of α- and γ-tocopherol. Mol Aspect Med 28:668–691
Rice C, Scolding N (2007) Strategies for achieving and monitoring myelin repair. J Neurol 254:275–283
Rimbach G, Minihane AM, Majewicz J, Fischer A, Pallauf J, Virgli F, Weinberg PD (2002) Regulation of cell signalling by vitamin E. Proc Nutr Soc 61:415–425
Roosendaal SD, Moraal B, Vrenken H, Castelijns JA, Pouwels PJ, Barkhof F, Geurts JJ (2008) In vivo MR imaging of hippocampal lesions in multiple sclerosis. J Magn Reson Imaging 27:726–731
Rose RC, Bode AM (1993) Biology of free radical scavengers: an evaluation of ascorbate. FASEB J 7:1135–1142
Rus H, Cudrici C, Niculescu T, Niculescu F, Shin ML (2006) Oligodendrocyte cell death in pathogenesis of multiple sclerosis: Protection of oligodendrocytes from apoptosis by complement. J Rehab Res Dev 43:123–132
Sailer M, Fischl B, Salat D, Tempelmann C, Schonfeld MA, Busa E et al (2003) Focal thinning of the cerebral cortex in multiple sclerosis. Brain 126:1734–1744
Saporito MS, Wilcox HM, Harptence KC, Lewis ME, Vaugth JL, Carswell S (1993) Pharmacological induction of nerve growth factor mRNA in adult brain. Expl Neurol 123:295–302
Saporito M, Brown E, Hartpence K, Wilcox H, Vaught J, Carswell S (1994) Chronic 1, 25-dihydroxyvitamin D3-mediated induction of nerve growth factor mRNA and protein in L929 fibroblasts and in adult rat brain. Brain Res 633:189–196
Schewe T (1995) Molecular actions of ebselen—an anti-inflammatory antioxidant. Gen Pharmacol 26:1153–1169
Scolding N (2001) Regenerating myelin. Brain 124:2129
Setzu A, Ffrench-Constant C, Franklin RJM (2004) CNS axons retain their competence for myelination throughout life. Glia 45: 307–311
Sim FJ, Franklin RJM, Hinks GL (2000) The re-expression of the homeodomain transcription factor Gtx during remyelination of experimentally induced demyelinating lesions in young and old rat brain. Neurosci 100:131–139
Smith KJ, Blakemore WF, McDonald WI (1979) Central remyelination restores secure conduction. Nat 280:395–396
Smith KJ, Kapoor R, Felts PA (1999) Demyelination: the role of reactive oxygen and nitrogen species. Brain Pathol 9:69–92
Traber MG, Atkinson J (2007) Vitamin E, antioxidant and nothing more. Free Radical Biol Med 43:4–15
Vercellino M, Plano F, Votta B, Mutani R, Giordana MT, Cavalla P (2005) Grey matter pathology in multiple sclerosis. J Neuropathol Exp Neurol 64:1101–1107
Wolf BB, Green DR (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem 274:20049–20052
Woodruff RH, Franklin RJM (1998) The expression of myelin basic protein exon 1 and exon 2 containing transcripts during myelination of the neonatal rat spinal cord—an in situ hybridization study. J Neurocytol 27:683–693
Woodruff RH, Franklin RJM (1999) Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: A comparative study. Glia 25:216–228
Zhang SC, Ge B, Duncan ID (1999) Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity. Proc Natl Acad Sci USA 96:4089–4094
Zhao C, Fancy SPJ, Kotter MR, Li WW, Franklin RJM (2005) Mechanisms of CNS remyelination—the key to therapeutic advances. J Neurol Sci 233:87–91