

The Effect of Caffeine on the Myelin Repair Following Experimental Demyelination Induction in the Adult Rat Hippocampus

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Abstract

Multiple sclerosis is characterized by the loss of oligodendrocytes and demyelination of axons. In this study, the effect of caffeine on spatial memory in rats was investigated following demyelination induction by lysolecithin (LPC). The expression of Myelin Basic Protein (MBP), Glial Fibrillary Acidic Protein (GFAP), and Olig2 (oligodendrocyte lineage marker) genes was also assessed in the hippocampus. Animals were divided into seven groups; control group: animals received normal saline by stereotaxic intrahippocampal injection in Dentate Gyrus (DG) area; LPC group: animals received 2 μ l lysolecithin by stereotaxic intrahippocampal injection in DG area (they were evaluated 7, 14, and 28 days after LPC injection; Caffeine- treated group: animals were treated with caffeine at doses of 30 mg/kg intraperitoneally for 7, 14, and 28 days after receiving LPC. Behavioral study was performed using Radial Arm Maze. Moreover, the RT-PCR was carried out for gene expression investigation. The demyelination and defective remyelination were noticeable on 28th day which suggests the demyelination decline caused by caffeine. Behavioral study showed that on the post-lesion days, the food finding time in the LPC group was significantly higher than that of the control group. Caffeine consumption significantly attenuated the food finding time in the treatment compared to the LPC group. The RT-PCR analysis indicated that the lysolecithin decreased the MBP expression especially on days 7 and 14 and conversely increased the Olig2 and GFAP expression. In addition, the caffeine enhanced the expression of MBP compared to that of the LPC group and reduced the Olig2 and GFAP expressions. Our results demonstrated that caffeine could increase the remyelination process in hippocampus and improve the spatial memory following demyelination induction by the LPC.

Keywords: Demyelination, Remyelination, Lysolecithin, Hippocampus, Caffeine

Introduction

Multiple sclerosis (MS) is a common autoimmune disorder of the central nervous system with a characteristic pathology that includes CNS demyelination and axonal damage, resulting in recurrent impairment of brain function (Keegan and Noseworthy, 2002). Lysophosphatidylcholines (LPC) also called lysolecithins, are a class of chemical compounds derived from phosphatidylcholins, which are toxic for the myelinating cells of CNS (Dixon et al., 1996). LPC has long been employed to create experimental demyelination by inducing myelin breakdown and apoptosis of Oligodendrocytes (Wallace et al., 2003). Indeed, lysophosphatidylcholine was recently shown to induce the influx of T cells with robust activation of macrophage/microglia in CNS. Activated microglial cells secrete a variety of cytokines which inhibit proliferation and differentiation of oligodendrocyte progenitors (Vela

et al., 2002). Caffeine is one of the members of methylxanthines which is largely anti-inflammatory in nature (Horrigan et al., 2006). As a result of having lipophilic property, caffeine easily passes from the blood brain barrier and as an adenosine receptors antagonist, it has different and complicated behavioral and biochemical effects on the CNS (Smith et al., 2003). Caffeine affects on neurogenesis, survival and proliferation of neural cells especially in hippocampus (Wentz and Magavi, 2009). The adult hippocampus, a vital center for learning and memory, is extremely vulnerable to various insults and neurological diseases (Nakafuku et al., 2002). Remyelination is a process in which myelin sheaths are generated and stored around the demyelinated axons. However, myelin sheaths are shorter and narrower (Franklin and French-Constant, 2008) and dependent on proliferation, migration, and differentiation of endogenous progenitor (Franklin, 2002).

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Although two major cell types, oligodendrocytes and neurons, are directly engaged in remyelination, it is clear that astrocytes and microglia are also involved in the myelin damage (Frederick and Miller, 2006 ; Zhang et al., 2001).

Astrocytes play an important role in pathological conditions of the nervous system. Accumulation of glial fibers is the histological landmark of the astrocyte response to CNS injury, appropriately named reactive gliosis. Such response is characterized by intense astrocyte proliferation (Bignami and Dahl, 1995).

Glial fibrillary acidic protein (GFAP) is an intermediate neurofilament expressed in the astrocytes and its levels were evaluated as a marker of astrocytes activity (McDonald and Ron, 1999). Oligodendrocytes are the myelinating cells of the CNS and are essential for proper brain function. These cells develop from an oligodendrocyte progenitor cells (OPC) (Raff et al., 1983) which arise in the subventricular zones and migrate extensively and differentiate into mature oligodendrocytes (Webb et al., 1995).

Olig2 (oligodendrocyte lineage marker) plays essential roles in oligodendrocyte specification and differentiation (Ligon et al., 2006) and is used in different studies as an oligodendrocyte precursor cells marker (Liu et al., 2007).

Myelin Basic Protein (MBP) is a major structural protein in myelin, thought to be primarily responsible for compaction and stabilization of the major dense line as well as playing a potent role in myelinogenesis (Jordan et al., 1989).

In the present study, a toxin-induced model of demyelination in the hippocampus of adult rat was used and the protective effect of caffeine on myelin repair and improvement of spatial memory following experimental demyelination by LPC was evaluated. Also, the gene expression for Olig2, MBP, and GFAP was assessed.

Materials and Methods

Animals

All experiments were carried out on adult male Wistar rats (Razi Institute, Karaj, Iran) weighting 180–200 g (8–10 weeks).

Animals were housed four per cage under a 12-h light/dark cycle in a room with controlled temperature ($23 \pm 2^\circ\text{C}$).

Food and water were available ad libitum. All the experiments were carried out according to the protocol approved by the Animal Ethics Committee of Urmia University, Urmia, Iran.

Stereotaxic lysolecithin microinjection and Treatments

After 1 week of acclimatization, animals were deeply anaesthetized with a mixture of ketamine hydrochloride and xylazine (Sigma, Germany) (10 and 2 mg/kg intra-peritoneal., respectively) and positioned in rat stereotaxic instrument (Narishige, Japan) in a skull-flat situation. After shaving the corresponding skull surface, using a bladed scalpel, a midline incision was made at the middle of shaved site and then rats were cannulated. Guide cannuls was prepared from the dentistry needles- head 23G which was located bilaterally in Dentate Gyrus (DG) area of hippocampus according to stereotaxic atlas (Paxinos and Watson, 2007) with the coordinates of (AP= -2.8) toward the bregma, (L=+1.8) toward the middle line and (DV= +2.8) from the level of skull. The process of myelin destruction was carried out by injection of 2 microliter lysolecithin 1% (Sigma, St. Louis, USA) in saline 0.9% with the rate of 1 $\mu\text{l}/\text{min}$ in DG area of hippocampus (Dehghan et al., 2012; Mozafari et al., 2011).

Animals were divided into seven groups; control group: animals received normal saline by stereotaxic intrahippocampal injection in Dentate Gyrus (DG) area; LPC group: animals received 2 μl lysolecithin (LPC) by stereotaxic intrahippocampal injection in DG area (they were evaluated 7, 14, and 28 days after LPC injection); Caffeine- treated group: animals were treated with caffeine at doses of 30mg/kg intraperitoneally for 7, 14 and 28 days after receiving LPC.

Behavioral testing

At the first day to habituate the rats to the new environment of the radial arm maze (RAM), they were individually placed inside the center of the maze and were given 5 min to explore the maze. Behavioral testing was performed 1 hour after caffeine injection. During the habituation day rats received no food available and the arms the rats visited were recorded to ensure they were visiting all arms of the maze.

The second and third days of training sessions which was consists of two sessions per day for 5 minutes in the morning and in the evening. An arm entry was counted when all four limbs of the rat were within an arm. At the end of this 2 day training session, the rats reached the learning criterion of 90% (Tarbali et al., 2013, MCGurk et al., 1989). In all groups the time of finding food was evaluated. Behavioral study was carried out during 3 periods so that the first period included the days 1-7 post LPC injection, the second period encompassed the days 12-18 and finally the third period included the days 22-28 after LPC

injection.

Tissue Preparation and Histological Assessment

Animals were re-anesthetized on day 28 post lesion and were perfused intracardially with 0.1 M phosphate buffered saline (PBS) and then with a solution of 4% paraformaldehyde in 0.1 M PBS (pH=7.4). The hemispheres were taken out and post fixed overnight in the same fixative at 4 °C. For paraffin embedding, tissues were first dehydrated in alcohol, cleared by incubations in xylene, and finally embedded in paraffin for 3 h, and blocked. Coronal serial sections (5 µm thickness) were obtained from the hemispheres using a rotary microtome and then were stained with 0.1 % Luxol Fast Blue (British Drug House, UK) solution at 60 °C for 3 h. Adequate contrast was made by transient immersion of preparations in 0.05 % lithium carbonate and 70 % alcohol. After distilled water washes, the sections were counter stained with 0.1 % Cresyl Fast Violet (Merck, Germany) for 4 min. Sections were washed in distilled water again and dehydrated in a graded series of alcohols, then cleared in xylene, cover slipped and the sites of demyelination were verified (Khezri et al., 2013).

Gene Expression Study

For gene expression study, animals were divided into control and experimental groups. After LPC injection, treatment groups received 30 mg/kg caffeine for 7, 14 and 28 days. At the end of mentioned days, animals were killed and the hippocampus was extracted and immediately preserved in liquid nitrogen. Total RNA was isolated (Barres, 1991), using TRIZOL reagent (Sigma Aldrich) according to the manufacturer's instructions. The final total RNA pellet was suspended in 30 µl of DEPC (diethylpyrocarbonate) –treated water (Fermentas). 5 µl of total RNA was used for spectrophotometric determination of the RNA concentration at 260 nm. For each sample, cDNA synthesis was performed using 1 µg of total RNA, Oligo-dT primer, M-MuL V reverse transcriptase and RNase inhibitor (Fermentas) based on the manufacturer's instruction. PCR was performed using 1 µl synthesized cDNA as template, specific primers and 2X PCR Master Mix (Cinnagen, Tehran) based on the manufacturer's instruction. Primer sequences for Olig2, GFAP, MBP and β-actin, were designed on the basis of the published sequences in Gen Bank (Table 1). Segments of Olig2, GFAP, MBP, and β -actin (internal control) cDNAs were amplified for 32, 25, 26 and 25 cycles, respectively. The reaction parameters were adjusted to obtain a condition with

linear relation between the number of PCR cycles and PCR products and with linear relation between the initial amount of cDNA template and PCR product. Ten microliters of amplified products was run on 1.5% agarose gel (Roche, Germany). Agarose gels were stained by ethidium bromide (0.5 µg mL⁻¹) and visualized under a UV light. A 50 bp DNA ladder (Gene Ruler 50-1000 bp, Fermentas) was used as a molecular size marker. Semi-quantitative analysis of PCR products were done by band densitometry using a computerized image analyzing system (Carestream Gel Pro 212 Imager, USA).

Table 1. Sequences of primers used for RT-PCR amplification

Gene	Primer	Primers sequence
MBP	Forward	5'-CCCTCAGAGTCCGACGAGCT-3'
	Reverse	5'-GCACCCCTGTCACCGCTA-3'
Olig2	Forward	5'-CTCTTTGGCTGAACACTCCAA-3'
	Reverse	5'-CCCAAAAAGGATTCCGTTCCA-3'
GFAP	Forward	5'-CTCGTGTGGATCTGGAGAGGAA-3'
	Reverse	5'-GCCCTCCAGCAATTTCTGTAG-3'

Statistical Analysis

The results are expressed as mean ± SEM. Data from molecular assessments were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc using SPSS statistical software. The averages obtained from behavioral assessments were compared by using analysis of variance for repeated measures. For all analysis, $P < 0.05$ was considered significant.

Results

Myelin Staining

Histological evaluation of hippocampus by using myelin staining showed a normal staining for the control animals. Lysolecithin induced an obvious demyelination in rat hippocampus and this effect was noticeable. Twenty eight days after lysolecithin injection, some demyelination was detectable whereas in the rats treated by caffeine, myelin staining was rather improved. Representative micrographs are shown in

Figure 1.

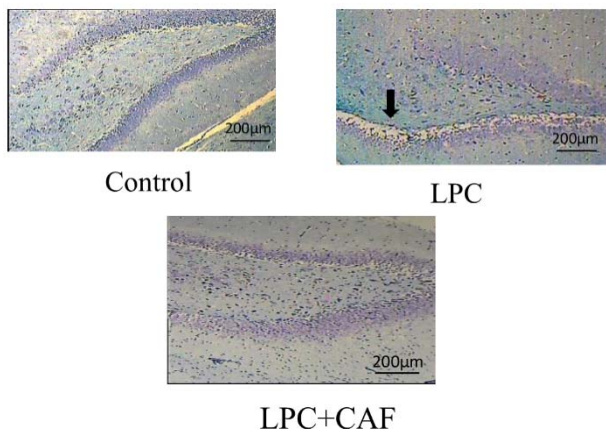


Figure 1. Representative micrographs showing the effect of LPC on demyelination and the effect of caffeine on myelin repair in the rat hippocampus DG area at day 28 post lysolecithin induced demyelination. LPC: lysolecithin-treated group, LPC+CAF: lysolecithin-treated animals injected with caffeine. Scale bar: 200 µm.

Behavioral test

The results obtained from behavioral study during first period (i.e. the days of 1-7 after LPC injection) showed that the food finding time in LPC group was significantly ($P<0.01$, $P<0.001$) longer than control. Food finding time in treatment group was higher than control and in the days 5th – 7th there is significant difference ($P<0.001$), although, in the days 5th – 7th, the food finding time in treatment group was significantly ($P<0.05$, $P<0.01$) lower than LPC group (Figure 2A).

The time for food finding in second period (i.e. the days 12-18) in LPC group was significantly ($P<0.05$, $P<0.001$) longer than control group. Treatment with caffeine, caused significant ($P<0.05$, $P<0.01$, $P<0.001$) reduction of food finding time in radial maze compared to LPC group. Although during this period the time for food finding in (LPC+CAF) group was higher than control but at 16th to 18th days there is no significant difference (Figure 2B).

In third period the food finding time in LPC group gradually decreased, however, it was significantly ($P<0.01$) longer than control. Caffeine consumption in treatment group during 22-28 days, significantly ($P<0.01$, $P<0.001$) attenuated the time for finding food compared to LPC group. Also, despite the time in treatment group was higher than control, but there is no significant difference (Figure 2C).

Expression levels of Olig2, GFAP and MBP

To evaluate demyelination and remyelination following LPC injection in hippocampus, we accomplished several gene expression studies on the lesion site at 7, 14, and 28 days post lesion using

semi-quantitative RT-PCR. Gel documentations revealed bands with different densities for Olig2, MBP and Glial fibrillary acidic protein (GFAP) genes in the experiment time course.

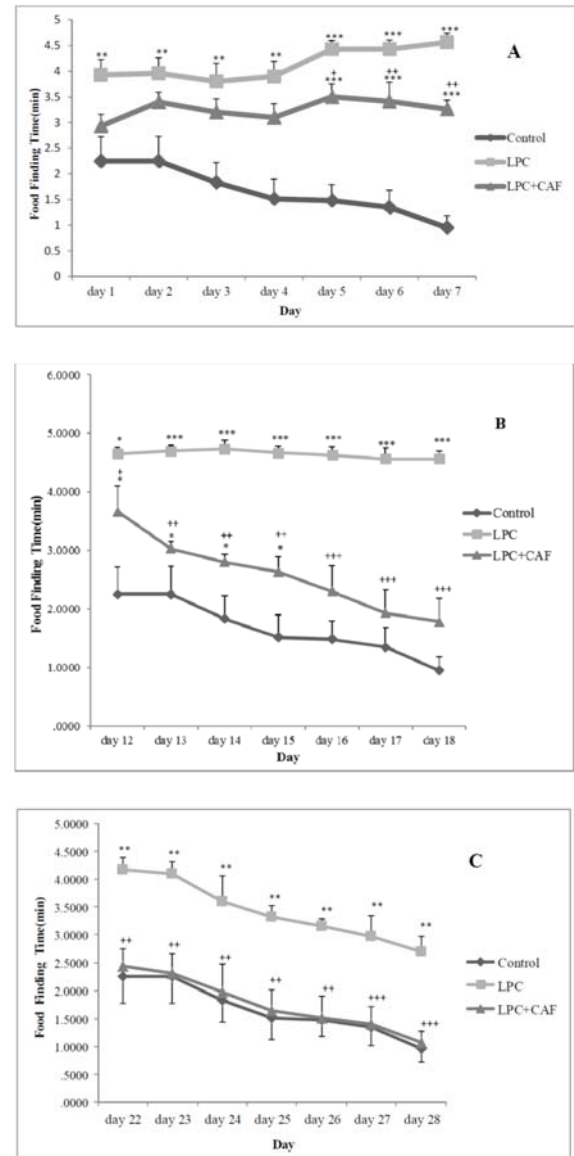


Figure 2. Impairment of hippocampal learning and memory following demyelination induction by LPC and caffeine injection in rats. (A) Food finding time at the first period (i.e.1-7 day post lesion) of behavioral test was delayed in both LPC (Lysolecithin) and LPC+CAF (Lysolecithin + Caffeine) groups compared to control. However, time in LPC+CAF group was lower than LPC group. (B) In second period (i.e. 12-18 day post lesion), time in LPC group was higher than treatment and control. (C) In third period (i.e. 22-28 day post lesion), there was no noticeable difference between LPC+CAF group and control. Data were expressed as mean \pm SE. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control, + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ compared to the LPC group (n=8).

Olig2 Gene Expression

Olig2 is used in different studies as an oligodendrocyte precursor cells marker (Liu et al., 2007). In the current study, Olig2 expression increased on 7 days post-lesion ($P<0.001$) and reached to the highest level on 14 days post-lesion ($P<0.001$) compared to the control group but it was decreased on the day 28 post-lesion, however, it still remained higher than control ($P<0.05$). The expression level of this gene in treatment group was significantly ($P<0.001$) lower than the LPC group all days post lesion (Figure 3A).

Glial fibrillary acidic protein (GFAP) Gene Expression

GFAP expression levels were evaluated as a marker of astrocytes activity (McDonald and Ron, 1999). The result of RT-PCR analysis showed that the lysolecithin significantly changed the GFAP expression level on days 7, 14 and 28 post lesion in patient groups to higher than control ($P<0.001$). This enhancement of GFAP level demonstrates astrocyte activity. Applying caffeine reduced the level of this gene in (LPC+CAF) group all days post lesion compared to LPC group ($P<0.01$, $P<0.001$) (Figure 3B).

Myelin Basic Protein (MBP) Gene Expression

MBP contributes to formation and compaction of myelin sheath (Baumann and Pham-Dinh 2001). The results showed that LPC injection caused significant reduction ($P<0.001$) in MBP gene expression on the day 7 and its mRNA level reached to minimum level on the day 14 post lesion in comparison to control ($P<0.001$). On the day 28, its expression was higher than days 7 and 14 but still it was significantly lower than control ($P<0.01$). These results signify hippocampus demyelination and myelinating cells depletion. Caffeine consumption significantly ($P<0.001$) increased MBP expression level in treatment groups compared to that of the LPC groups on the days 7, 14 and 28 post lesion. The day 28 had the highest level for MBP expression. Even on the day 28, its expression was significantly higher than control ($P<0.001$) (Figure 3C).

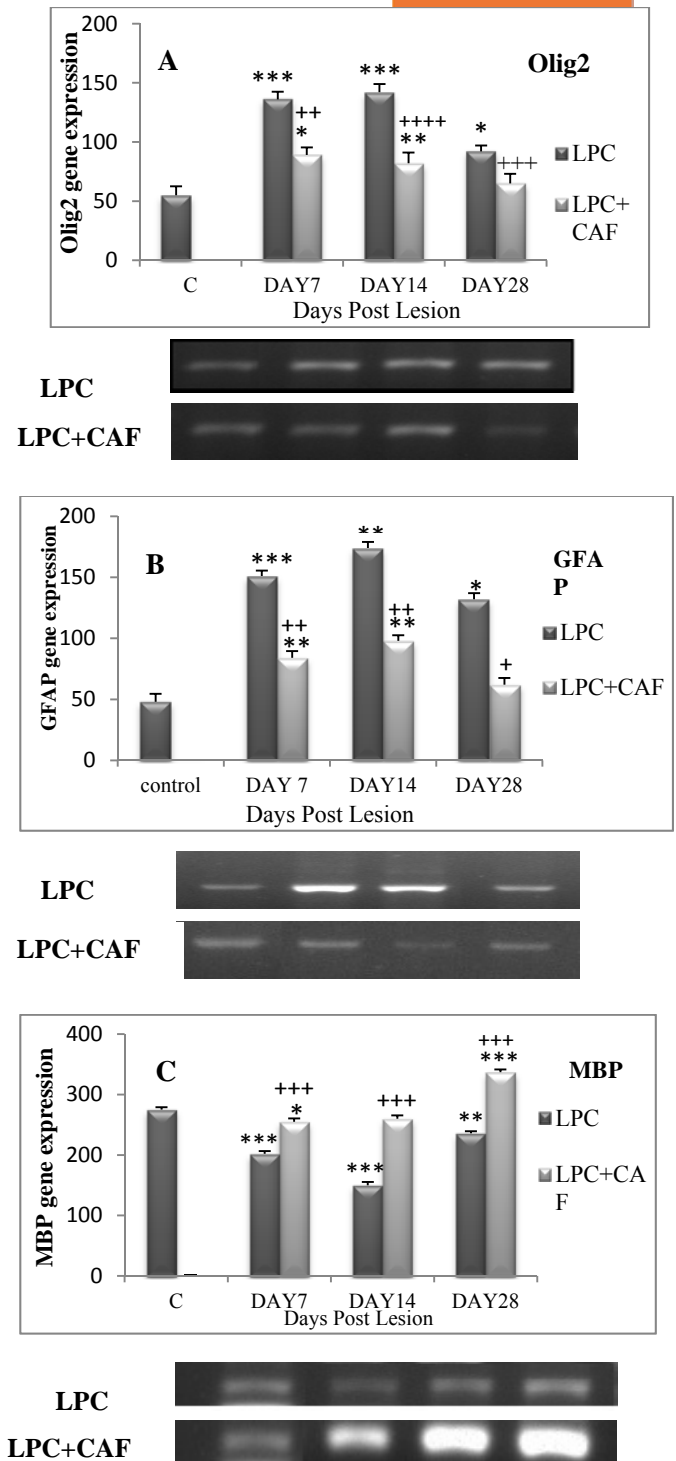


Figure 3. Assessment of gene expression in hippocampus. The gene expression level was assessed using RT-PCR and normalized to β -actin band density. (A) Changes in the expression of Olig2 as an oligodendrocyte precursor cell marker. (B) Changes in the GFAP expression following lesion induction in hippocampus. (C) Changes in the expression of myelin basic protein (MBP) gene following lysolecithin injection. * $P<0.05$, ** $P<0.01$ *** $P<0.001$ compared to control and + $P<0.05$, +++ $P<0.001$ compared to LPC group in the same day (n = 5). LPC (Lysolecithin) and LPC+CAF (Lysolecithin + Caffeine).

Discussion

Multiple sclerosis is one of the most common neurological disorders in young people with the age average of about 30 (Shivane and Chakrabarty, 2007). Inflammation and demyelination of CNS are its symptoms (Sherafat et al., 2012). Demyelination is the destruction of myelinating protein which forms a sheath around the axon of neurons. In the central nervous system, the myelin destruction process is occurred by the direct attack of immunity system to oligodendrocytes which from the myelin sheath and protect it, so, it can be a diagnostic aspect for MS (Tomassini and Pozzilli, 2009; Franklin and French-Constant, 2008).

Lysolecithin is an analogue of lysophosphatidylcholin and has a detergent effect with a special effect on myelinating cells. Hippocampus structure is known as one of the important gray substances which are affected by MS (Franklin and French-Constant, 2008). In this study, the effect of caffeine on memory recovery and also on remyelination was evaluated by expression measurement of Olig2, MBP and GFAP after lysolecithin intra hippocampus injection. Caffeine is one of the methylxanthin members which have anti-inflammatory property. In many neural destruction events it has been shown that the caffeine has neural protective effects (Franklin and French-Constant, 2008). This substance as adenosine receptor antagonist has many behavioral and biochemical effects in neural system (Wentz and Magavi, 2009).

The results obtained from our behavioral study at the first period showed that the time of finding food in LPC group was longer than control. However, all days of this period, the time for finding food in the caffeine-treated group was lower than LPC group. In second period, the food finding time in LPC group was longer than the first period and was also significantly ($P<0.05$, $P<0.001$) longer than control group. In these days, the LPC rats moved slowly in radial maze and they entered frequently in repetitive or empty of food arms. These cases show that the injection of lysolecithin in hippocampus led to demyelination in this area and also had the maximum destruction effect during these days. Thus, because of losing myelin sheath and the death of neurons and myelinating cells, the synaptic link between the neural cells in hippocampus encounters some problems and leads to memory and learning disorders. Makinodan and his colleagues (2008) showed that the lysolecithin led to special learning and memory disorder. They

suggested that after LPC injection, no maturity of oligodendrocytes was observed and it was indicative of the intensification of microglia and releasing the inflammatory factors and their provocative effects on the cell death of oligodendrocytes which led to delay in myelinating (Makinodan et al., 2008). The administration of caffeine in second period led to significant ($P<0.05$, $P<0.01$, $P<0.001$) decrease in the food finding time in treatment group compared to LPC group. Caffeine is known as a strong inhibitor of phosphodiesterase enzyme (Ribeiro and Sebastiao, 2010). Moreover, it leads to increase the level of cAMP by blocking the adenosine receptors. Cyclic AMP, by activating the route dependent on protein kinase A has anti-inflammatory effects and causes memory and learning reinforcement (Aandahl et al., 2002). In the third period, the time of finding food was decreased in the LPC group. Since no treatment was done in this group, this progressive reduction maybe due to the internal regeneration of hippocampus, of course, it had little effect on the improvement of memory disorder, because food finding time in this group was significantly ($P<0.01$) longer than the control and also the treatment groups. The time in the treated group with caffeine had no significant difference with control group. The rats treated with the caffeine were able to find the food in a shorter time and less errors. The findings showed that treatment with the caffeine has protective effects against the demyelination of neural cells and also causes to improve the spatial memory and the learning related to the hippocampus damage.

At the molecular level, the results showed that Olig2 gene expression in the LPC groups all days after LPC injection was significantly ($P<0.05$, $P<0.001$) higher than that of the control group. Olig2 belongs to a family of oligodendroglial transcription factors (Bignami and Dahl, 1995) and its expression is increased in reactivated oligodendrocyte precursor cells (Fancy et al., 2004). In our study, the observed Olig2 gene expression increase could reflect the existence of oligodendrogenesis potential in the demyelinated hippocampus. The expression level of this gene in treatment group was significantly ($P<0.001$) lower than LPC group all days post lesion and at day 28, there is no significant difference between control and treatment group. Dehghan and colleagues (2012) reported that the expression of Olig2 was increased on the days 7 and 13 post lesion, indicating immigration and activity of many oligodendrocyte precursor cells in demyelinating

area for generating the mature oligodendrocytes (Dehghan et al., 2012). Hence, lower expression of Olig2 in caffeine-treated groups implies more differentiation of precursor cells compared to LPC group.

GFAP levels are regulated under the pathological condition. The high regulation of GFAP is one of the important characteristics of astrocytes which is usually seen after the CNS lesion. Therefore, the study on GFAP regulation is useful for recognizing and evaluating the neurological and pathological problems (Gomes et al., 1999). The results of RT-PCR analysis suggest that the consumption of lysolecithin could increase the GFAP expression level on the days 7, 14, and 28 post lesion in the LPC group compared to that of the control group. The enhancement of GFAP level indicates astrocyte activity. Caffeine reduced the mRNA level of this gene in LPC+CAF group all days post lesion compared to the LPC group. The level of adenosine is increased in the CNS in pathological condition which in turn leads to enhancement of astrocyte activity and proliferation through the adenosine receptors and astrogliosis production through the metabolisms dependent on free radicals (Allaman et al., 2003). The long term effects of active astrogliosis lead to secreting the toxic and inflammatory factors and also bring about an inflammatory condition in the lesion area (Allaman et al., 2003; Pittock and Lucchinetti, 2007). Therefore, it is likely that the caffeine inhibits the adenosine activity under pathological condition by blocking the adenosine receptors especially A2A in the CNS and thus reduces the releasing of toxic and inflammatory factors which lead to the apoptosis of neural system. Mozafari and colleagues showed that the expression level of GFAP after the injection of lysolecithin in the optic chiasma was maximum on the days 2th and 7th and it decreased on 28th day, although in this day, GFAP was higher than control. They reported that the astrocytes activity in damaged area is increased in response to the LPC induced demyelination and thus increased the GFAP expression (Mozafari et al., 2011).

MBP, a component of myelin proteins essential for myelin compaction and stability in CNS (Goudarzvand et al., 2010), is frequently used as an index of myelination (Goudarzvand et al., 2010; Messersmith et al., 2000). In the current study LPC injection caused to a significant reduction ($P < 0.01$) in MBP expression all days post lesion compared to the control. Lowest expression level of MBP mRNA was observed on the day 14. These results demonstrate a hippocampus demyelination

and myelinating cells depletion. Afterwards, a significant MBP increase was observed on the day 28 that suggests an endogenous repair. The behavioral and histological observations in this study support this fact. The partial endogenous repair of myelin in this report might be due to recruitment, migration, and differentiation of adjacent OPCs or neural stem cells.

Caffeine consumption in treatment group significantly ($P < 0.001$) increased MBP expression level compared to the LPC groups on the days 7, 14 and 28 post lesion. However, on the day 28 reached to the highest level compared to control. Perhaps newly differentiated oligodendrocytes express higher amount of MBP compared to older ones (Messersmith et al., 2000). As it was indicated in the behavioral test, the caffeine-treated group had the best function in the third period (i.e. the days 22 to 28) which reflects remyelination and a better learning in those days. The remyelination process requires the mature oligodendrocytes derived from the progenitor cells of the CNS which is located under the ventricular area. This observation may be due to accelerated process of OPCs (Oligodendrocyte Progenitor Cell) proliferation and migration towards the lesion (Menn et al., 2006; Dehghan et al., 2012) and also due to in advanced differentiation of OPCs to myelinating oligodendrocytes. OPCs have widespread distribution throughout the gray and white matter (Zhang et al., 1999; Setzu et al., 2004). The previous reports indicate caffeine can affect increase of proliferation, migration and differentiation of progenitor cells in hippocampus (Wentz and Magavi, 2009).

Conclusion

According to the obtained results, we can conclude that caffeine could attenuate the lysolecithin pathology at behavioral, histological, and molecular levels. Our findings may provide a neurological basis for the epidemiological investigation on the relation between caffeine consumption and the progression of MS in humans.

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