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Research Report

Microinjection of L-arginine into corpus callosum cause reduction in myelin concentration and neuroinflammation

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ABSTRACT

Role of nitric oxide (NO) in inflammationary diseases such as multiple sclerosis (MS) has been proposed previously. We sought to examine if NO plays centrally a key role in MS related phenomena; demyelination or neuroinflammation. Female Wistar rats (weighing 200–250 g) were mounted in a stereotaxic apparatus and received injections of L-arginine aimed at corpus callosum (AP: 1.2, L: ±1.8, V: 3.2). The drug (50–200 μg/rat) was microinjected intra-corpus callosum repeatedly (3–5 times/each per day). Control groups solely received saline (1 μg/rat) into the corpus callosum. The animals were tested for the novelty seeking behavior using the conditioning task. Memory impairment was examined using the shuttle box and Y-maze. L-NAME was pre-injected to L-arginine to involve the NO. All animals' brains were also processed for histological evaluation. L-arginine produced significant changes in the novelty seeking behavior but not in the memory formation, evidenced by passive avoidance and alternation behaviors. Pre-injection of L-NAME reversed the response to L-arginine. Present study further revealed a prominent inflammation as well as myelin elimination in the L-arginine treated rats' brains. These data suggest that the NO infusion in the myelin rich areas such as corpus callosum may lead to MS signs centrally.

1. Introduction

Multiple sclerosis (MS) is identified an autoimmune, inflammatory, demyelinating, and neurodegenerative disease of the central nervous system affecting over 2 million people worldwide (Kurnellas et al., 2007). The main histopathological hallmarks of MS are listed as inflammation, demyelination, oligodendrocyte death, gliosis, axonal damage and neurodegeneration (Bruck and Stadelmann, 2005; Prat and Antel, 2005). The disease, though the pathogenesis of MS is unknown, represents an autoimmune disorder directed against nervous system antigens (Hemmer et al., 2002; Noseworthy et al., 2000; Wekerke, 1998). The underlying mechanism of neural damage in MS has often been addressed by use of animal model called experimental autoimmune encephalomyelitis (EAE) (Hohlfeld, 2009; Kurnellas et al., 2007). EAE shows similarity in pathological,

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histological and clinical features to MS and covers different aspects of the disorder (Gold et al., 2006; Subramaniam, 2005). EAE is induced in some animal species and strains either by immunization with myelin components or by passive transfer of encephalotigenic T-cells.

Nitric oxide (NO) is a free-radical produced from the oxidation of terminal guanidino nitrogen of arginine, and this reaction is catalyzed by a NADPH-dependent enzyme, NO synthase (NOS) (Lowenstein and Snyder, 1992). This diffusible neuronal second messenger, NO, is centrally formed by a neuronal isoform of the NOS (nNOS) (Knowles and Mocada, 1994), the enzyme that is blocked by N^G-nitro-L-arginine methyl ester, L-NAME (Moncada et al., 1991). The molecule NO is involved in MS (Bishop et al., 2009; David et al., 2006; Gold et al., 1997); a continuous and high concentration of NO metabolites in CSF and serum of MS patients in relapsing phase is suggested to cause damages to myelin and oligodendroglia (Acar and Idiman, 2003). A level of NO is considered to be a useful marker of the MS disease (Danilov and Andersson, 2003).

Although evidence (Encinas et al., 2005) points to the contribution of NO in various aspects of the disorder such as inflammation, oligodendrocyte injury, changes in synaptic transmission, axonal degeneration and neuronal death (Encinas et al., 2005), the pathogenic role of NO in MS, however, remains controversial. The present study discovers the importance of NO in a highly myelinated area corpus callosum (CC) by investigating the disease process in the L-arginine injected female Wistar rats both behaviorally and histologically.

The female rats were preferred based on the previous data showing that the MS occurs more commonly in females than males and that the prevalence of the disease is much greater in the gender (Pozzilli et al., 2003; Whitacre et al., 1999). It should be notified that no significant differences in brain lesions of MS has been identified during ovarian cycle or change in ratio of progesterone/estradiol levels in women (Bansil et al., 1999; Holmqvist et al., 2006).

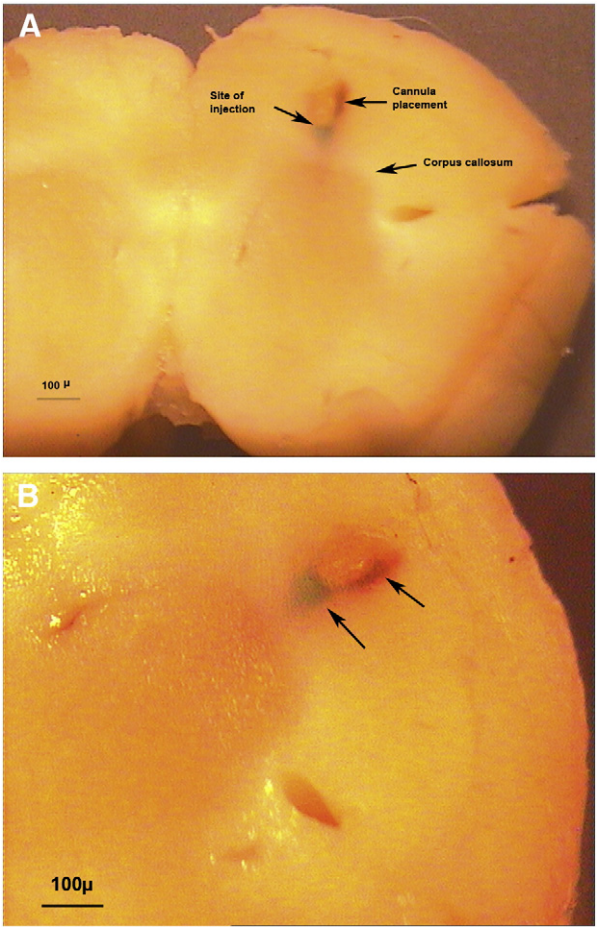

Fig. 1 – (A) Cannulae placements in corpus callosum as evidenced by ink injection in a volume of 1 µl/rat by using the same set up as used for intra-central amygdala injection of drugs (AP: 1.2). Scale shows the magnification and the arrowheads signify the point of microinjection, the cannula placement, and the corpus callosum, (B) The Fig. 1(A) under the magnification.

2. Results

2.1. *Verification of site of microinjection*

Site for microinjections was revealed by injection of 1 µl of a methylene blue solution into the corpus callosum using the same injection set up as used for microinjecting of the drugs (Fig. 1).

2.2. *Effects of microinjection of L-arginine into the corpus callosum on neuroinflammation and demyelination*

Light microscopic observations revealed a decrease ($F_{6,28}$= 9.636, p<0.001) in the quantity of cell population in L-arginine treated animals (Fig. 2E–F) in comparison with the controls (Fig. 2A–C). A significant decrease in myelin concentration was observed ($F_{6,28}$=13.611, p<0.01) in the samples administered L-arginine (Fig. 2G) compared with that of controls (Fig. 2D). The effect of neuroinflammation at the site of injections was also appeared due to infiltration of the cells (Fig. 2H).

Pre-injection of L-NAME (50–200 µg/rat, into the corpus callosum) to the L-arginine caused an attenuation or completely blockade on the effects of L-arginine indicating the NO involvement in the processes (Fig. 2I–J).

2.3. *Dose–response of L-arginine in novelty seeking behavior as evidenced using conditioning place preference paradigm*

Administration of L-arginine at different doses (50–200 µg/rat, into the corpus callosum) after five times resulted in a significant change in behavior response in comparison with the control (saline treated group: 1 µl/rat, into the corpus callosum) ($F_{3,20}$=10.673; p<0.001, Fig. 3). More analysis of the data showed that L-arginine at 100 µg/rat potentiated the signs of the behavior when was injected into the corpus callosum for five times. However, the injection of the lower or higher doses of the agent resulted in a decrease in the response signifying the bell shaped response of the NO precursor.

2.4. *Dose–response of L-NAME pre-injection to L-arginine in novelty seeking behavior*

Microinjection of L-NAME (50–200 μg/rat, into the corpus callosum) prior to the administration of L-arginine (100 μg/rat, into the corpus callosum, five times) resulted in a significant change in response compared with the control, solely L-arginine (100 μg/rat, into the corpus callosum, five times) treated group ($F_{3,20}=26.673$; $p<0.0001$, Fig. 4). Post-hoc analysis showed a decrease in the response at all doses of the antagonist.

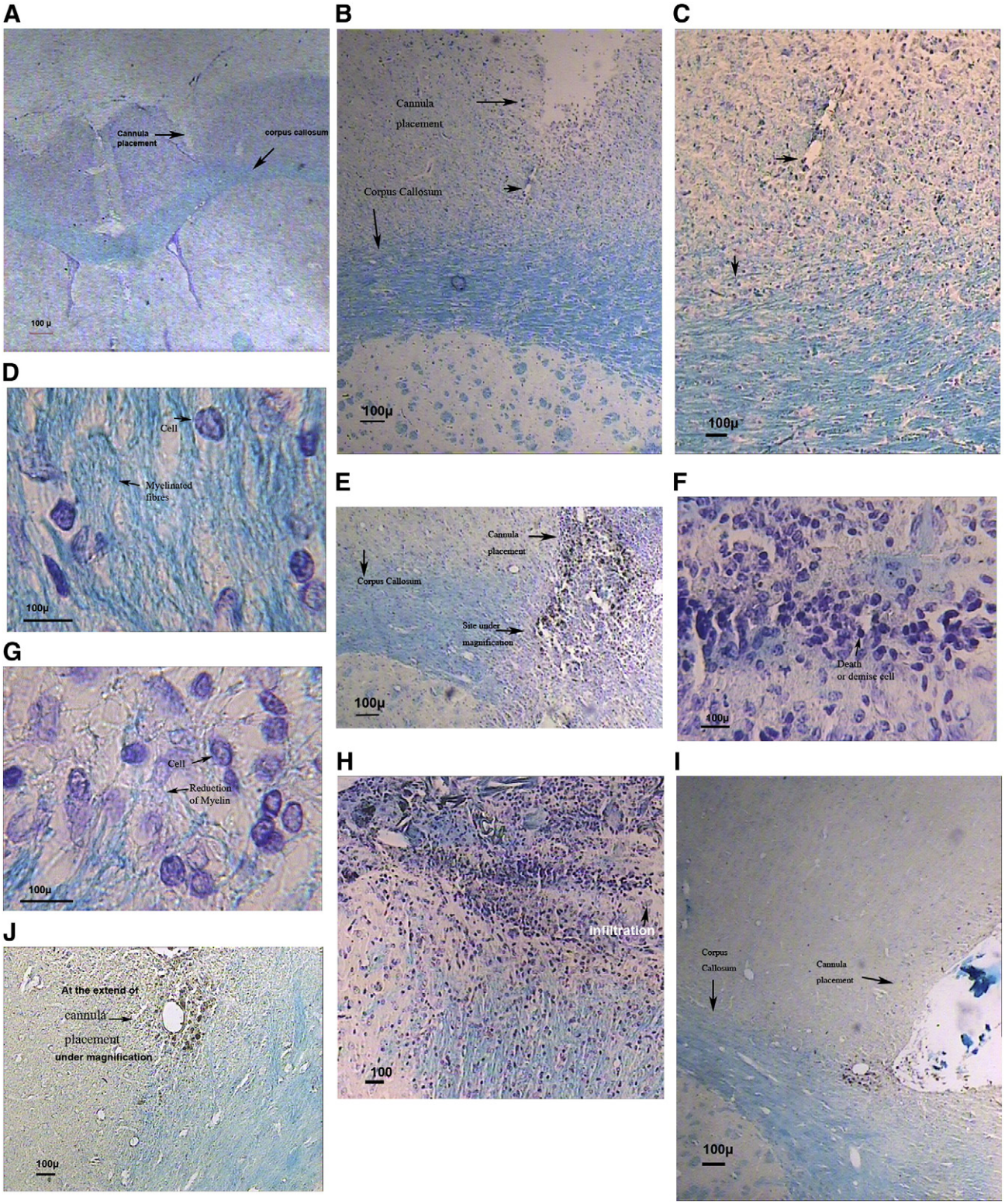

Fig. 2 – Reduction of the cell population, demyelination, and neuroinflammation in brain slices from the rats which received L-arginine (50–200 μg/rat, into the corpus callosum) (E–H). No evidence for these signs either in controls (A–D) or in L-NAME pre-injected slices was found (I–J). Arrows denote the effects and the comparisons.

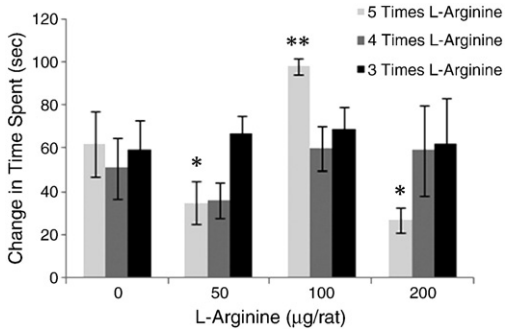

Fig. 3 – Response to L-arginine (50–200 μg/rat, 3-5 times, into the corpus callosum) treatment in the novelty seeking program. The rats were drug microinjected and experienced a paradigm for the novelty seeking behavior detailed in the Experimental procedure. Control group simply received saline into the corpus callosum (1 μl/rat, 3-5 times), but, the group was trained for the behavior using the same protocol. *$p<0.05$ and **$p<0.01$ different from control according to the post-hoc analysis (Tukey–Kramer).

2.5. *Measurement of memory impairment due to L-arginine (intra-corpus callosum) using shuttle box and Y-maze*

Examining memory in rats injected with different doses of L-arginine (50–200 μg/rat, into the corpus callosum) using both shuttle box and Y-maze, showed that there was no significant change in their response in comparison with the control groups ($p>0.05$).

3. Discussion

There are many controversies regarding the role of nitric oxide (NO) in demyelination of the central nervous system. While some studies suggest that nitric oxide ameliorates the demyelinating diseases such as EAE (O'Brien et al., 1999), the others point at the aggravating effects of NO in demyelination (Garthwaite et al., 2005; Liñares et al., 2006; Moncada and Bolaños, 2006). To further investigate if direct application of NO precursor into CNS of normal rats results in a myelin content change, we have injected L-arginine as a NO precursor (50–200 μg/rat) into a highly myelinated area, corpus callosum. This approach which has not been taken previously, show that there is a significant reduction in myelin content of the corpus callosum proven by both histological and behavioral examinations. The changes in novelty behavior indicated as dependent on doses of L-arginine when the agent was injected more frequently (five times). However, blocking of NO producing due to L-NAME pre-injections, during the task, attenuated or completely blocked the effects, indicating that NO release plays a role in learning, novelty behavior formation (Katzoff et al., 2002). It is hypothesized that there is a relation between corpus callosum damage and cognitive dysfunction in MS (Calabrese and Penner, 2007). Additionally, it is expressed that the performance of the majority of neuropsychological tests requires preservation of cognitive networks and of their connections and that the corpus, because of its crucial role in interhemispheric transfer of different stimuli, is likely to play a crucial role in this (Mesaros et al., 2009). Also, corpus callosum as a white matter containing area has been previously shown to be associated with cognitive impairment and therefore been considered as an early structural marker for the children at risk of cognitive difficulties (Pride, 2010). The present results with a regarding to the histological data provided by L-NAME pre-injection to L-arginine propose that NO overload in corpus callosum may contribute as a risk factor for loss of axon transport and learning. In accordance, the role of NO in learning damage is supported by findings that cognitive dysfunction can occur at all stages of MS (Chiaravalloti and DeLuca, 2008). However, to clearly define the association between corpus damage and bell shaped effect of the L-arginine microinjection in novelty seeking behavior, and the relationship between this area atrophy and the performance of the task remain elusive at the molecular level. Despite inducing a learning impairment, L-arginine treatment did not affect the memory in these animals suggesting that (1) the brain areas involved in memory such as hippocampus have not been affected by local infusion of the L-arginine and (2) the mild damage to neuronal structures in corpus callosum is insufficient to induce memory malfunctioning by itself; therefore, long-term treatment may be essential to cover more areas.

The histological analysis of luxol fast blue stained corpus callosum of the L-arginine treated rats and those of pre-injected by L-NAME indicates that NO mediates a damage to both neuron and myelin structures. Hypomyelination in different areas of the CNS with unknown etiology, mostly in the periventricular white matter, has also been reported to be involved in multiple sclerosis (Landro et al., 2000). The effect of NO on myelin sheath damage may be a result of lipid peroxidation which may indirectly affect the protein components of the membrane as well. Bizzozero et al. (2004) have shown that exposure of rat optic nerves to NO results in myelin decompaction, accompanied by S-nitrosylation of a cysteine-rich proteolipid protein.

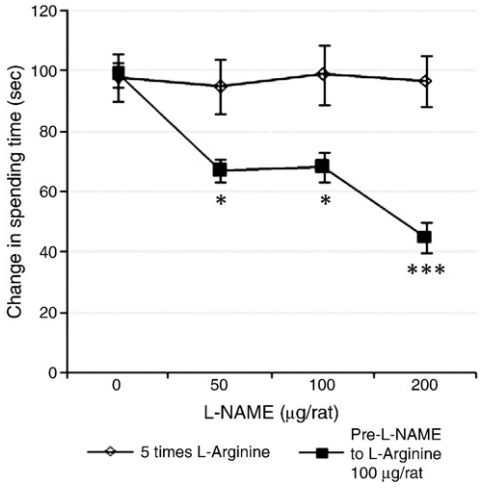

Fig. 4 – Pre-injection of L-NAME in L-arginine treated rats as detailed in the Experimental procedure. Significant effects were resulted statistically at all doses of the antagonist. *$p<0.05$ and ***$p<0.01$ different from control (treated single L-arginine at100 μg/rat, into the corpus callosum).

Pathological analysis of the L-arginine treated rat brains were also indicative of neuroinflammation (Fig. 2H), suggesting that NO triggers inflammatory factors as indicated elsewhere (Brenner et al., 2001). In our future approaches, molecular mechanism(s) involved in histological and behavioral changes induced by NO would be addressed. Nevertheless, we suggest that NO overproduction in CNS may be a potential risk factor in neurodegeneration.

4. Experimental procedures

4.1. Subjects

Adult female Wistar rats weighing 200–250 g (Pasteur Institute, Tehran, Iran) were housed in standard plastic cages (two in each) in a standard temperature (21±3 °C) and 12-h light/dark cycle (lights on at 07:00 a.m.) and with food and water ad libitum. The experiments were carried out during the light phase of the cycle. Each animal was tested once, and six animals were used per subgroup. They were killed with an overdose of chloroform at the end of each experiment. All experiments were carried out in accordance with the National Institutes of Health Guide for care and use of laboratory animals (NIH Publication No. 80-23, revised 1996) and approved by local committee of ethics at Shahed University (Document No. 7941, 2010). A total of 154 rats were included in the study. The animals were categorized into different L-arginine dose groups for receiving the microinjection of the NO agent into the corpus callosum; each group was further divided into subgroups based on the times repeatedly receiving the drug (three to five times/once a day). Control groups were only injected saline (1 μg/rat) in accordance with the protocol. A maximum of 126 rats were taken both for microinjectioning and the novelty seeking behavior testing. About 28 rats were both microinjected and treated memory tests (the shuttle box and Y-maze). All the animals' brains were examined histopathologically. More than 12 rats were excluded of the study because of the incorrect site cannulation.

4.2. Drugs

L-Arginine (Sigma Chemical Co., USA) was prepared in sterile 0.9% NaCl solution and was bilaterally injected into the area of interest (corpus callosum, CC) at desired doses with a final volume of 1 μl/rat. Control animals received 0.9% physiological saline as vehicle (1 μl/rat). Injections of Ketamine (100 mg/kg i.p.) with Xylazine (20 mg/kg i.p.) purchased from Veterinary Organization of Iran were used to anesthetize the animals prior to their placement in stereotactic apparatus and for perfusion.

4.3. Stereotaxic surgery

The animals were anesthetized and placed in a stereotax apparatus, with the incisor bar set at approximately 3.3 mm below the horizontal zero to achieve a flat skull position. An incision was made to expose the rat skull. Two holes were drilled in the skull according to stereotaxic coordinates: AP=1.2 mm anterior to bregma, L=±1.8 mm, and V=3.2 mm (Paxinos and Watson, 2005). Two guide cannulae (21 Gauge) were inserted into the holes. To do the bilateral injections in corpus callosum, the guide cannulae were lowered 2.5 mm below bregma through the above mentioned drilled holes. Using a jeweler's screw and dental cement, the guide cannulae were anchored, fixed and covered, respectively. After the surgery, dummy inner cannulae were inserted into the guide cannulae and left in place until the time of injections. All animals were allowed to recover for 1 week before starting the microinjections of L-arginine.

4.3.1. Injection into the corpus callosum

The animals were gently restrained by hand; the dummy cannulae were removed from the guide cannulae. L-Arginine (50–200 μg/rat, into the corpus callosum) was injected repeatedly from three to five times/once a day. Injections were made directly into the nucleus through the guide cannulae by using a 27-Gauge injecting needle connected to a 5.0-μl glass Hamilton syringe using a polyethylene tubing (0.3 mm internal diameter). The injecting needles projected 0.7 mm outside from the tip of the guides. Injections for all groups were made over a 30-s period, and the injection cannulae left in the guide cannulae for an additional 60-s to facilitate the diffusion of the drug. Each drug dose was tested only once in six animals.

4.4. Novelty seeking behavior using conditioning place preference apparatus and paradigm

4.4.1. Apparatus

A two compartment conditioned place preference (CPP) apparatus (30×60×30 cm) was used in these experiments. Place conditioning was conducted using an unbiased program, with minor modifications to a previously described design (Karami et al., 2002; Zarrindast et al., 2002). The apparatus was divided into two equal-sized compartments, by inserting a removable wall in the middle. Both compartments were colored white, but they were striped black differently (vertical vs. horizontal). The compartments were also distinguishable by texture and olfactory cues. To provide the tactile difference between the compartments, one had a smooth surface but the other was ridged. To provide the olfactory difference one compartment was odored by a drop of natural aqueous rose extract placed in a corner of the compartment. Rats displayed no consistent preference for any of the compartments before the starting of the injections, indicating the unbiased used procedure. All experiments were recorded using an Ethovision system equipped with a videocamera located 120 cm above the apparatus. The files were then blindly reviewed by an observer.

4.4.2. Paradigm

The paradigm consisted of three phases.

Familiarization phase. On day 1, animals participated in a habituation session. They were placed in the middle line of the apparatus with the removable wall raised 12 cm above the floor and allowed free access to the entire apparatus for 15 min. After the completion of the phase the animals were randomly assigned to the L-arginine dose (50–200 μg/rat) treatment groups (n=6 per group); they were drug microinjected and transported to the experimental room. Control

group solely received saline (1 μg/rat, into the corpus callosum).

Conditioning phase. This phase was started a day after the first phase lasted 3–5 days; the animals assigned to L-arginine dose groups received placements into one of the compartments for 45 min with the partitioning wall installed; half of them were placed into one and half received placements into other compartment as been described previously (Bardo et al., 1989). Placements occurred once daily after that the animals were drug microinjected and transported to the experimental room. Administration of saline in the control groups was conducted using the same protocol as for the experimental animals.

Testing phase. Test sessions were carried out a day after the last microinjectioning session. The animals were tested only once under the same conditions as provided in the habituation phase. The time spent for the novel compartment relative to the familiar side was then assessed after reviewing the video files recorded by ethovision system.

A difference in the duration spent in familiar side which the animals were placed in, through the conditioning phase, relative to the other compartment was measured to quantifying the novelty behaviors.

4.5. *Passive (shuttle) avoidance test*

The shuttle box (BPT Co., Tehran: 20×80×20 cm) consisted of two compartments as been explained previously (Kim et al., 2009; Mirshekar et al., 2010); one contained a safe-illuminated 100 W bulb; the other equipped with 2 mm stainless steel rods spaced 1 cm apart, and compartments were separated by a guillotine door. Each rat was first placed in the shuttle box to adapt for 5 min. The animal was then exposed to conditioned stimulus (tone of lights) and unconditioned stimulus (1 mA, 1-s scrambled foot shock). For the acquisition trial, the rats were initially placed in the illuminated compartment while the light of the compartment kept off, and the door separating them was opened 120 s later. The light got on to measure the time that each rat spent to enter to the other compartment considered as initial latency (IL). When animals entered the dark compartment, the door automatically was closed and a shock (1 mA, 1 s) was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, the rats were again placed in the illuminated compartment for the assessment of the retention trials; the time taken for a rat to enter the dark compartment after opening the door was measured as "Step through latency" (STL) in the trial.

4.6. *Y-maze test*

Memory impairment was tested using a Y-maze after the last administration of L-arginine or vehicle. The Y-maze with three horizontal arms (with 40 cm long, 15 cm wide, and 30 cm high walls) located symmetrically with 120° angles relative to each other was constructed from Plexiglass as been previously detailed (Kim et al., 2009; Mirshekar et al., 2010). Animals were initially placed in one arm and consequently in the others (i.e., ABCAB, etc.). The number of arm entries for each animal over an 8-min period was then recorded manually. An actual alternative was also defined as entries into all three arms in consecutive choices (i.e., ABC, CAB, or BCA but not BAB). Maze arms were thoroughly cleaned between the sessions to remove residual odors. The percentage of alternations was considered as an index for an active memory and calculated by using the following equation: (number of alternations/total arm entries − 2) × 100.

4.7. *Experimental design*

4.7.1. *Induction and assessment of novelty seeking behavior*

The effects of administration of L-arginine (50–200 μg/rat, into the corpus callosum) on induction of novelty seeking behavior in animals were determined using the conditioning protocol as mentioned elsewhere in Experimental procedure (see Section 4.4.).

4.7.2. *Effects of L-arginine injections into the corpus callosum on learning and memory using shuttle box and Y-maze*

In these sets of experiments, after injections of different dose of L-arginine (50–200 μg/rat, into the corpus callosum) the animals were analyzed for the tests. The responses were calculated to assess the impairment effect induced of the drug on memory.

4.7.3. *Effects of micro injection of the nitric oxide agent, L-arginine, into the corpus callosum on neuroinflammation and demyelination*

To determine the effects of L-arginine (50–200 μg/rat, into the corpus callosum) on neuroinflammmation and demyelination, the agent was bilaterally administered using the injection set up as mentioned in detail in Experimental procedure. The animals' brains were sectioned and verified using the protocol of luxol fast blue by the protocol followed.

4.8. *Histology*

4.8.1. *Verification of the cannulae's place*

Cannulae were located according to the specifications by Paxinos and Watson (2005). After completion of behavioral testing, animals were killed with an overdose of chloroform. Histological verification of the inserted cannulae was done by injecting ink (0.5 μl of 1% aquatic methylene blue solution) into the guide cannulae, using 27-Gauge injection cannulae that projected 1.8 mm further ventral to the guide's tip. Brains were then removed and fixed in 10% formalin solution for 48 h and then sectioned from the cannulae inserted brain areas (Fig. 1). Data from rats with injection sites located outside the appropriate area were excluded from the statistical analyses.

4.8.2. *Histological study by luxol fast blue staining*

Luxol fast blue is commonly used to observe myelin under the light microscopy. The stain works via an acid–base reaction, that is, lipoprotein in myelin is replaced by the dye base and therefore a color change resulted. The myelin fibers turn to blue and the nerve cells to purple (Sheehan and Hrapchak, 1980).

To perform the luxol fast blue staining, brains were dissected from the chloroform killed animals and fixed in sucrose 4% buffered formaldehyde for 24 h. After being fixed, samples were embedded either in paraffin or Tissue-tek for frozen sectioning. Sections of 5–6 μm were then mounted on glass slides greased by

albumin and retained at room temperature for about 24 h. Frozen sections were de-fatted by directly being placed into 1:1 alcohol/chloroform for a few hours followed by 95% ethyl alcohol. Both paraffin and frozen sections were left in luxol fast blue (Merck Co., Germany) solution in 56 °C oven overnight, rinsed off excess stain with 95% ethyl alcohol followed by washes in lithium carbonate (0.05%)/ethanol 70%/distilled water for differentiation. Then they were counterstained in the cresyl violet (Merck Co., Germany) solution for 30–40 s. The slides were then dehydrated in 95% ethanol and in 100% ethanol for 5–10 min, cleared in xylene and mounted with entallen glue (Merck Co., Germany).

4.9. *Statistical analysis*

All behavioral results are expressed as mean of time (sec) ± SEM (standard error of mean). To do the comparisons, one-way analysis of variance (ANOVA) followed by appropriate post-hoc analysis (Tukey–Kramer) were used. *p*-values less than 0.05 were considered to be statistically significant.

To quantify the histology data, the stained brains' slices of the treated animals by the luxol fast blue were examined micrographically by the video-light microscope (Olympus) at the site of injections; areas of 100 μm^2 were defined as units of quantification measurements and analyzed by Image Tool program (UTHSCSA ImageTool, version 2.03). This program after the spatial and density comparing using the calibrations obtained by the control samples provided the data ± SEM at the significant levels.

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