INTRAHIPPOCAMPAL ADMINISTRATION OF VITAMIN C AND PROGESTERONE ATTENUATES SPATIAL LEARNING AND MEMORY IMPAIRMENTS IN MULTIPLE SCLEROSIS RATS

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ABSTRACT

It seems antioxidant and sex hormones are able to protect the multiple sclerosis (MS) rats against spatial memory reduction. Since sex hormones and oxidative stress are affective in the process of multiple sclerosis (MS), as well as cognitive functions, the study evaluates the effects of intrahippocampal injection of vitamin C and progesterone, alone or in combination on spatial memory in multiple sclerosis. Sixty-three (63) male Wistar rats were divided into nine groups (n = 7): control, (saline), sesame oil, lesion (ethidium bromide (EB)), vitamin C (1, 5 mg/kg), progesterone (0.1, 1 µg/µl) and combination therapy. In combination therapy, animals were treated with vitamin C (5 mg/kg) + progesterone (0.01 mg/kg). Animals in experimental groups received different treatments for 7 days. Characteristics of learning and spatial memory were assessed using Morris Water Maze (MWM). The results showed that intrahippocampal injection of ethidium bromide destroys MWM significantly (p<0.05). Vitamin C (5 mg/kg), progesterone (0.1 mg/kg) and vitamin C (5 mg/kg) + progesterone (0.1 mg/kg) significantly decreased latency time and travelled distance (P<0.05) in MS or lesion rats. In comparison with control group, the lesion group decreased and progesterone 0.1 mg/kg + vitamin C 5 mg/kg increased the time and distance in the target quadrant after the platform was removed. In comparison with lesion group, vitamin C (1 and 5 mg/kg), progesterone (0.1 and 1 mg/kg) and vitamin C + progesterone effective doses increased the time and distance in the target quadrant after the platform was removed. The results showed that multiple sclerosis rats had a decreased travelled distance and time spent in target quadrant to find the hidden platform in a MWM task. Vitamin C and progesterone alone improved spatial memory in comparison to lesion group. Effective doses of vitamin C + effective dose of progesterone had more improving effect on memory.

Keywords: Neuroscience, Neurosteroid, Antioxidant, Demylination, Progesterone, Learning and memory impairments, Multiple sclerosis rats

INTRODUCTION

Multiple sclerosis (MS) is a multifocal inflammatory disease of the brain and the spinal cord. The main cause of MS is unknown, although genetic and environmental factors have been shown to contribute to its etiology (Sospedra and Martin, 2005). As in many autoimmune diseases there is a higher prevalence of women than men in MS, with a female – male ratio of 2.6:1. This ratio is rising with a disproportional increase of females, especially in the relapse-onset form of the disease (Alonso and Hernan, 2007; Debouverie *et al.,* 2008). Sex hormones are affective in the process of MS disease, for example there is

evidence that estrogen has inhibitory effects on MS disease (Ito *et al.,* 2001). Also during pregnancy in woman suffering from MS, because of the high level of estrogen and progesterone, the severity of the disease is reduced, but after delivery the clinical symptoms were aggravated (Confavreuex *et al.,* 1998). The finding suggests that MS is influenced by sex hormones. Clinically, progesterone produced a moderate delay of disease onset and reduced the clinical scores. Thus, progesterone attenuated disease severity, and reduced the inflammatory response and the occurrence of demyelination in the spinal cord during the acute phase of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis characterized by demyelination and immune cell infiltration in the spinal cord (Garay *et al.,* 2007). In the spinal cord, progesterone increases motor neuron survival after axotomy or injury, protects cultured neurons against glutamate toxicity and normalizes defective functional parameters of injured neurons (Labombarda *et al.,* 2002). In conjunction with neuronal effects, progesterone strongly influences myelin synthesis in the peripheral and central nervous system (Melcangi *et al.,* 2000). In the oligodendrocytes, the central myelin producing glia, progesterone increases myelination in culture and in cerebellum, as shown by the increased expression of myelin basic protein (MBP) (Ibanez *et al.,* 2003; Schumacher *et al.,* 2004). On the other hand, in MS disease, the axon of nerve fibres is damaged. Axonal damage often produces oxidative stress, nitric oxide, dysfunction of sodium/potassium pump, neurotoxicity induced by glutamate and destruction of myelin's protective agents (Dutta and Trapp, 2007). It has been determined that progesterone has antioxidant and anti-glutamatergic effect. Progesterone is able to induce the re-expression of sodium/potassium pump in axon. Progesterone down regulates the myelin basic protein (MBP) which is an index of axonal damage (Stein, 2008). Other antioxidant such as ascorbic acid (vitamin C) is an essential micronutrient required for normal metabolic functioning of the body (Gey, 1998). Ascorbic acid is a potent antioxidant, which is highly concentrated in the central nervous system (Sanchez-Moreno *et al.,* 2003). In most studies, researchers believed that ascorbic acid prevents memory deficit by its antioxidant effect (Castagne *et al.,* 2004). Since it is widely accepted that cognitive dysfunction occurs in 40 – 70% of MS patients (Sartori and Edan, 2006) and the most common cognitive deficits are memory dysfunction and spatial perception impairment (Glanz *et al.,* 2012), so the aim of present study was to determine the alone treatment of ascorbic acid and progesterone and co treatment of them on spatial memory task in MS induced rats. On the other hand the major question is whether vitamin C, progesterone alone or in combination has the ability to alter spatial memory in male MS rats or not?

MATERIAL AND METHODS

Animals: Sixty three (63) adult male Wistar rats weighing 200 - 250 g were housed in standard hygienic plastic cages under a 12 hour light/dark cycle (lights on at 07:00 a.m.) in a room with controlled temperature (23 ± 2 °C). Food and water were available *ad libitum*. The experiments were carried out during the light phase of the cycle. All animal procedures were performed according to the National Institutes of Health's Guide for the care and use of laboratory animals.

Experimental Demyelination with Ethidium Bromide (EB) and Treatments: The animals were randomly divided into nine groups (7 animals per group): group 1 - control, (no treatment); group 2 - sham, (sesame oil, solvent of progesterone); group 3 - saline (solvent of vitamin C); group 4 - (ethidium bromide, or lesion group); group 5 - (ethidium bromide + 1 mg/kg vitamin C) group 6 - (ethidium bromide + 5 mg/kg vitamin C), group 7 - (ethidium bromide + 1 mg/kg progesterone); group 8 - (ethidium bromide + 0.1 mg/kg progesterone), group 9 - (ethidium bromide + 1 mg/kg progesterone +5 mg/kg vitamin C). For the surgical demyelination procedure, the animals were anaesthetized with intraperitoneal injection of ketamine (100

mg/kg) and xylazine (20 mg/kg) and placed on the rat stereotaxic instrument (Stoelting, USA) in the skull-flat position. Hair of the corresponding skull surface was shaved and then, an incision was made to expose the skull. Two holes were drilled in the skull according to appropriate coordinates to achieve cornu ammonis (CA1) of hippocampal formation (3.8 mm dorsal to the bregma, 2.4 mm deep from the dorsal surface and ± 2.2 mm laterality) (Paxinos and Watson, 1986). Two guide cannulae (21 gauges) were inserted into the holes and fixed using dental cement. After the surgery, dummy inner cannulae were inserted into the guide cannulae and left in place until the injections were made. All animals were allowed to recover for 1 week before starting the microinjections. Demyelination was induced bilaterally by direct single injection of 3µl of 0.01% ethidium bromide (EB) in sterile 0.9% saline (Goudarzvand *et al.,* 2013). Animals in experimental groups 4 – 9 received vitamin C or progesterone and vitamin C + progesterone with above mentioned doses for 7 days post lesion (Hooshmandi *et al.,* 2011). The animals from groups 2 and 3 were injected equal volume of sesame oil or sterile 0.9% saline. Injections for all groups were made at the rate of 1µl/minute using a 10-µl Hamilton syringe, and the needle was kept in the guide cannulae for an additional 60 second in order to facilitate the diffusion of the drug.

Morris Water Maze Test: The Morris water maze was black circular pool (136 cm in diameter and 100 cm in height). The pool was filled to a depth of 60 cm with water (20 ± 1 °C) and divided into four quadrants of equal area (NE, SE, SW and NW). A platform (10 cm in diameter) was centred in one of the four quadrants of the pool and submerged 1 cm below the water surface so that it was not visible at water level. The swimming was monitored by a video camera, which was positioned directly above the centre of the pool. The pool was located in a test room, which contained various prominent visual cues (Moosavi *et al.,* 2006).

One week after surgery, the rats were trained in the water maze. The single training session consisted of eight trials (in two blocks) with four different starting positions that were equally distributed around the perimeter of the maze. The task requires rats to swim to the hidden platform guided by distal spatial cues. After mounting the platform, the rats were allowed to remain there for 20 seconds, and then were placed in a holding cage for 30 seconds until the start of the next trial. Rats were given a maximum of 60 seconds to find the platform and if it failed to find the platform in 60 seconds, it was placed on the platform and allowed to rest for 20 seconds. Latency to platform and distance travelled were collected and analyzed. After completion of the training, the animals were returned to their home cages until retention testing 24 hours later. The probe trial consisted of 60 seconds free swim period without a platform and the time swum in the target quadrant was recorded. In order to assess the possibility of drug interference with animal sensory and motor coordination or the animal motivation, the capability of rats to escape maximum of 60 seconds to find the platform and if it failed to find the platform in 60 seconds, it was placed on the platform and allowed to rest for 20 seconds, the capability of rats to escape to a visible platform was tested in this study. Latency to platform and distance travelled were collected and analyzed later. After completion of the training, the animals were returned to their home cages until retention testing 24 hour later. The trained rats were given four trials for visual-motor coordination on the visible platform (Castagne *et al.,* 2004; Mohaddes *et al.,* 2009).

Data Analysis: SPSS 13.0 software was used for statistical comparisons of data and data were expressed as means ± SEM. For comparisons between Block 1 and Block 2 in each group, a paired-sample t-test was used. The statistical analysis of the data between groups was carried out by one-way ANOVA followed by Turkey test. In all comparisons, $p<0.05$ was the criterion for statistical significance.

RESULTS AND DISCUSSION

In comparison of block 1 and block 2, vitamin C (5 mg/kg), progesterone (0.1 mg/kg) and vitamin C (5 mg/kg) + progesterone (0.1 mg/kg) significantly decreased latency time ($p<0.05$) and travelled distance ($p<0.05$) in MS or lesion rats (Figures 1 and 2). Probe test data were compared between groups. One-way ANOVA of the distance travelled in the target quadrant revealed significant differences ($p<0.05$) between groups. In comparison with control group, the lesion group decreased and progesterone 0.1 mg/kg + vitamin C 5 mg/kg increased the time and distance in the target quadrant after the platform was removed. In comparison with lesion group, vitamin C (1 and 5 mg/kg), progesterone (0.1 and 1 mg/kg) and vitamin C + progesterone effective doses increased the time and distance in the target quadrant after the platform was removed (Figures 3 and 4). No treatments significantly changed swimming speed in the target quadrant.

In the present study vitamin C, progesterone, and vitamin C + progesterone were used for the first time in evaluating memory impairment of ethidium bromide (EB) - induced multiple sclerosis (MS). The results showed that MS rats had decreased travelled distance and time spent in target quadrant to find the hidden platform in a Morris Water Maze task. Vitamin C (1 and 5 mg/kg), progesterone (0.1 and 1 mg/kg) and vitamin C + progesterone effective doses (vitamin C 5 mg/kg + progesterone 0.1 mg/kg) administration improved the acquisition and retrieval in MS rats. Ethidium bromide induced focal demyelination by selectively damaging glial cells, which include oligodendrocytes (central nervous system myelin forming cells) and astrocytes (Spanevello *et al.,* 2009). Several studies have proposed that demyelinating insults occur in the central nervous system gray matter of MS patients. Hippocampal formation is known as one of the important gray matters which are reported to be affected by MS (Geurts *et al.,* 2007). Using this model, the study found that direct single injection of a 0.01% EB solution into the cornu ammonis (CA1) of hippocampal formation impaired hippocampal-dependent spatial learning and memory performances. Analyses of swimming velocity to reach the hidden platform revealed no differences between experimental groups, disproving any non-specific effects of EB microinjection on spatial acquisition and memory. These results demonstrated that the impairing effects of gliotoxin microinjection on spatial learning and memory were not due to any non-specific fluctuations in gross motor activity or motivational state. In this study progesterone in both doses significantly improved the spatial memory in comparison to MS or lesion group. In the field of progesterone effects on memory, there are different and sometimes paradoxical reports. May be some factors such as the model of administration, behavioural test kind, gender, age of animal, time of hormone treatment, and dose of hormone can induce different results. For example, it has been shown that progesterone alone or in combination with estrogen, improved scopolamine-induced impairment of working memory and reference memory as effectively as estrogen supplementation.

Estrogen and progesterone improved scopolamine-induced impairment of spatial memory (Tanabe *et al.,* 2004). In another study, it has been found that levels of progesterone appeared to be tied to verbal memory and global cognition among women who were in early post menopause, and the higher the levels of progesterone, the better the outcomes on tests of verbal memory and global cognition in these younger women (Henderson *et al.,* 2013). In another report, long-term treatment with estrogen or estrogen + progesterone (3 months, or 10 months after ovariectomy) significantly enhanced acquisition of the memory by aged animals after long-term loss of ovarian function in female Sprague-Dawley rats (Gibbs, 2000). These findings suggested that repeated treatment with estrogen and progesterone initiated within a specific period of time after the loss of ovarian function may be effective at preventing specific negative effects of hormone deprivation on brain aging and cognitive decline (Gibbs, 2000).

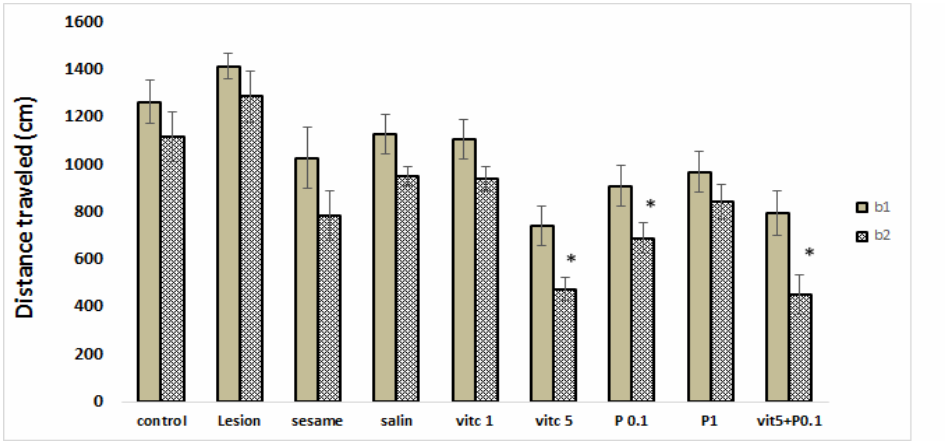

Figure 1: Effects of vitamin C and progesterone on the travelled distance to find hidden platform in two consecutive blocks (b1 and b2) in MS (or lesion) rats. Data represent means ± SEM (n=7),*p<0.05, significantly different when compared with the b1 same group

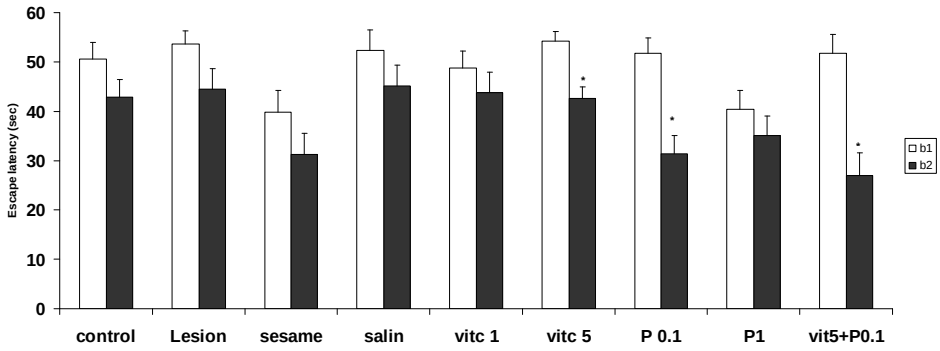

Figure 2: Effect of vitamin C and progesterone on the escape latency to find hidden platform in two consecutive blocks (b1 and b2). Data represent means ± SEM (n=7), *P<0.05, significantly different when compared with the b1 same group

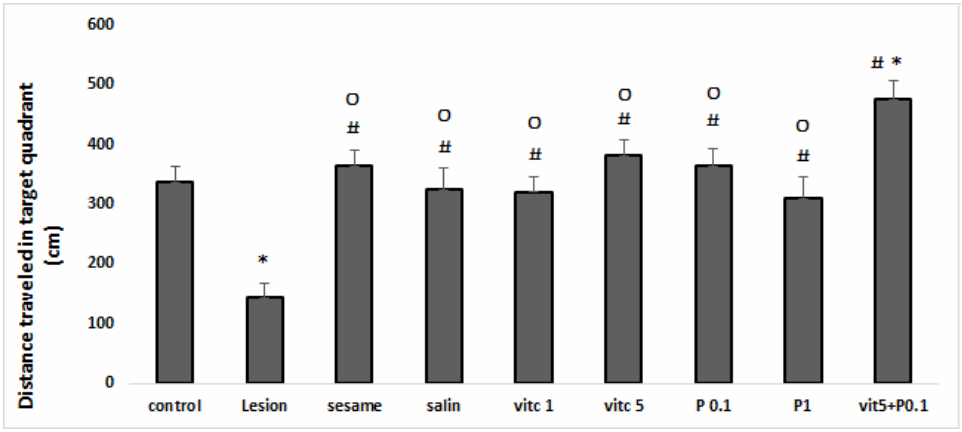

Figure 3: Effect of vitamin C and progesterone on the travelled distance in trial sessions of the Morris water maze test. Data represent means ± SEM (n=7), *p<0.05 significantly different when compared with the control group. # P<0.05 significantly different when compared with the lesion group, O p<0.05 significantly different when compared with the effective dose group

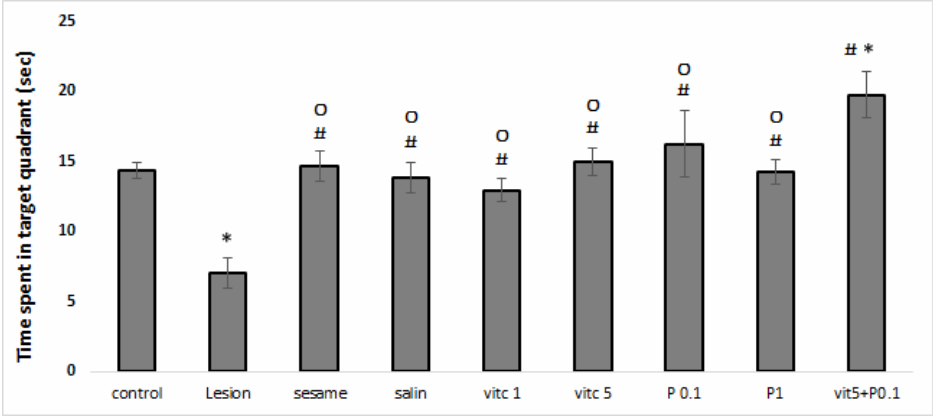

Figure 4: Effect of vitamin C and progesterone on the escape latency (or time spent in target quadrant) in trial sessions of the Morris water maze test. Data represent means ± SEM (n=7), *P<0.05 significantly different when compared with the control group, # p<0.05 significantly different when compared with the lesion group, O p<0.05 significantly different when compared with the effective dose group

It has been reported that acute progesterone treatment (subcutaneous injections of progesterone at 500 microgram) impaired spatial working memory in intact male and female rats. These results suggested that acute progesterone treatment interferes with spatial working memory consolidation, but not recognition (non-spatial) working memory. As such, the observed sexual incongruities in progesterone's effects on working memory suggested that progesterone-based hormone therapies have a negative impact on cognition (Sun *et al.,* 2010). In another study it has been reported progesterone supplementation reversed the cognitive enhancing effects of ovariectomy. This result suggested that whereas ovariectomy of the aged female rat enhanced learning and the ability to handle numerous items of spatial working memory information, progesterone was detrimental to this aspect of performance (Bimonte-Nelson *et al.,* 2004). Also El-Bakri *et al.* (2004) indicated that progesterone treatment in ovariectomized rats did not show significant learning compared to the vehicle treated groups in a Morris water maze task. It has been indicated that progesterone up-regulates the mRNA and protein expression of neuronal BDNF in the injured spinal cord and also BDNF protein in the normal tissue.

Concomitantly, steroid treatment also prevented the lesion-induced chromatolysis, supporting at the molecular and morphological levels the neuroprotective actions of progesterone (Gonaza *et al.,* 2004). A growing list of publications also gives evidence of the protective and trophic effects of progesterone. In the PNS, progesterone promotes myelination (Azcoitia *et al.,* 2003) and this stimulatory effect can be extended to the CNS. Indeed, progesterone stimulates myelination in organotypic slices cultures of 7-days-old (P7) rat and mouse cerebellum (Ghoumari *et al.,* 2003) and partially reverses toxin-induced demyelination in old male rats (Ibanez *et al.,* 2004). Progesterone also facilitated cognitive recovery and prevents neurodegeneration after cortical contusion (Stein, 2001). Finally, increased stability of BDNF protein and mRNA will result from the inhibition of oxidants and free radicals arising after spinal cord injury, since progesterone prevents injury-induced lipid peroxidation (Roof *et al.,* 1997) and exerts antioxidants effects in a murine model of spinal cord neurodegeneration (Gonzalez-Deniselle *et al.,* 2003). The mechanisms involved in the neuroprotective effects of progesterone are still not completely understood. However, it is known that the hormone has antioxidant properties (Roof *et al.,* 1997), regulates the expression of trophic factors such as brain-

derived neurotrophic factor (Gonzalez-Deniselle *et al.,* 2007), elicits the activation of intracellular signalling pathways involved in the promotion of cell survival (Singh, 2005), increases the expression of antiapoptotic molecules such as Bcl-2 and Bcl-XL, and reduces the expression of proapoptotic molecules such as Bax, Bad and caspase-3 (Yao *et al.,* 2005). Some of these effects of progesterone may be mediated by the activation of classical progestin receptors, which are widely expressed in the brain (Guerra-Araiza *et al.,* 2003). According to these reports, progesterone has different effects in memory including enhancement, no effect or decrease of memory formation. Taken together, these observations highlight the fact that progesterone is perhaps determinants of memory. In this study progesterone improved spatial memory in MS rats.

Also according to the results of study, intrahippocampal microinjection of vitamin C increased the distance travelled in target quadrant (increasing the spatial memory). Shahidi *et al.* (2008) indicated that both short-term and long-term supplementation with ascorbic acid had facilitatory effects on acquisition and retrieval processes of passive avoidance learning and memory in rats. It has been reported that, ascorbic acid could reduce the risk of dementia caused by aging, or prevent memory impairment due to the scopolamine (Parle and Dhingra, 2003) and homocyctein (Reis *et al.,* 2002). Also, it has been reported that local applications of ascorbic acid enhanced the response of neurons to dopamine and glutamate (Rebec and Pierce, 1994). Glutamate is a neurotransmitter which has a critical role in learning and memory processing. Therefore it is possible that part of the ascorbic acid effects may be due to its neurotransmitter modulator functions. Several studies have demonstrated that vitamin C is neuroprotective in adult animal models of hypoxic-ischemic injury (Wang, 2000), ascorbic acid injection is considered to inhibit necrotic cell death by suppression of calpain activation. In a study it has been showed that intraventricular vitamin C injection had a neuroprotective effect against hypoxic-ischemic brain injury in neonatal rats. Vitamin C reduced the percent brain damage, macroscopic brain injury and the number of necrotic cells. Vitamin C also inhibited calpain activation associated with necrotic cell death after hypoxic-ischemic in neonatal rat brain (Miura *et al.,* 2006). Also it has been reported that vitamin C restores acetyl cholinesterase activity that has an essential role in learning and memory (Ambali *et al.,* 2010).

Conclusion: The data from this study showed that vitamin C and progesterone were capable of protecting MS rats against spatial memory reduction. Although this was predictable based on antioxidant and protective effects of these substances but more experimental data, and in particular more information about the actions and effects of progesterone and vitamin C in multiple sclerosis are necessary for the development of more targeted and efficient steroid plus antioxidant treatments.

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