

Research Article

## Effect of Intrahippocampal Administration of Vitamin C and Progesterone on Learning in a Model of Multiple Sclerosis in Rats

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### Abstract

**Purpose:** The purpose of this study was to evaluate the effect of intrahippocampal injection of vitamin C and progesterone, alone or in combination, on passive avoidance learning (PAL) in multiple sclerosis.

**Methods:** Sixty- three male wistar rats were divided into nine groups (n=7) as following: control (saline), lesion, vitamin C (0.2, 1, 5 mg/kg), progesterone (0.01, 0.1, 1 µg/µl) and combination therapy. Lesion was induced by intrahippocampal injection of ethidium bromide. In combination therapy, animals were treated with vitamin C (5 mg/kg) plus progesterone (0.01 mg/kg). Animals in experimental groups received different treatments for 7 days, and then all groups were tested for step through latency (STL).

**Results:** Our results showed that intrahippocampal injection of ethidium bromide destroys PAL significantly (p<0.001). Treatment with vitamin C (5mg/kg) significantly (p<0.05) improved PAL. Lower doses of progesterone did not affect latency but dose of 1 µg/µl significantly (p<0.05) increased STL. In combination therapy group STL was significantly (p<0.05) more than in the lesion group, although it was not significantly different from the vitamin C group.

**Conclusion:** Based on our results, we concluded that intrahippocampal injection of vitamin C improves memory for PAL, but progesterone alone or in combination with vitamin C had no improving effects on memory.

### Introduction

Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS) in which the myelin sheet of the nerve fibers is destroyed.<sup>1</sup> Myelin is a unique component of the nervous system which increases the efficiency and speed of action potential through the nerve cells.<sup>2</sup> Without myelin to protect neurons, the brain and spinal cord signals that allow us to interact with the environment malfunction.<sup>3</sup>

Hippocampus is the center of learning and memory in the central nervous system. This area is very sensitive and vulnerable in neurological diseases and ischemia<sup>4</sup> and severely affected by oxidative damage.<sup>5</sup> Dentate gyrus (DG) of the hippocampus, because of its neural stem cells has the ability of restoration and proliferation of cells in rodents and humans throughout life.<sup>6</sup> Cognitive impairment occurs in more than 65% of patients with MS, and usually their ability to recall previously learned information reduces.<sup>3</sup> Due to the high vulnerability of the hippocampus and the capability of reconstruction, it is the appropriate area for studying the mechanisms involved in this process.

Antioxidants have a protective role in neurons (inhibition of apoptosis) and are involved in nerve regeneration.<sup>7</sup> Ascorbic acid (Vitamin C) is a low molecular weight antioxidant that scavenges the reactive oxygen species

(ROS) through electron transfer rapidly and prevents lipid peroxidation.<sup>8</sup> While it reduces lipid peroxidation, it increases catalase enzymatic activity in the brain, which has the compensatory mechanisms in the formation of free radicals.<sup>9</sup> The important role of oxidative stress of the hippocampus is shown in passive avoidance memory deficits.<sup>10</sup> Vitamin C is also an essential element to promote myelination.<sup>11</sup> Asymmetric distribution of vitamin C in different regions of the brain indicates its vital role in the brain. Vitamin C is transmitted through sodium-dependent vitamin C transporter type 2 (SVCT2) into neurons and the brain<sup>12</sup> and the neuronal protective role of vitamin C is in relation to the essential role of this transporter.<sup>13</sup> SVCT 2 is found more frequently in dense regions of the neurons in the brain such as the hippocampus, cortex and cerebellum where the issue is related to the regional distribution of ascorbate in the brain. Ascorbate participates in the synthesis of collagen, which is associated with the formation of myelin.<sup>12</sup> Vitamin C also can boost learning, memory, and impede memory deficiencies in different experimental conditions.<sup>14</sup> It has positive effects on acquisition and retrieval procedure of passive avoidance learning and memory in rats.<sup>15</sup>

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Sex hormones affect the MS disease process.<sup>16</sup> Progesterone is a possible factor to boost myelination by oligodendrocytes in the CNS.<sup>2</sup> Progesterone has different effects on neurogenesis, regeneration, myelination, recovery from traumatic brain injury and inflammation. Progesterone receptors are located in regions that are involved in fear, stress and anxiety<sup>17</sup> such as the hippocampus.<sup>18</sup> In regards to progesterone receptors, they are located in the dorsal hippocampus, location of DG, which may have an important role in different aspects of memory.<sup>19</sup>

Steroid hormones affect different functions of the brain such as mood, cognition, reproductive and motor behavior in a sex-dependent process, and this gender difference exists in cognitive areas like neocortex, hippocampus and amygdala. On the other hand, it is reported that progesterone has variant effects such as being ineffective, worsening or improving of disease.<sup>20</sup> Progesterone modulates the improvement effect of estradiol-induced on memory retention.<sup>21</sup> Steroid hormones also attenuate peroxide production and leakage of free radicals from mitochondria of brain and reduce oxidative stress.<sup>22</sup> Sex steroids decrease ROS via scavenging these products in the brain.<sup>2</sup>

To further substantiate the effects of vitamin C and progesterone on memory, intrahippocampal administration of vitamin C or progesterone alone or in combination was investigated in ethidium bromide-induced MS in rats.

## Materials and Methods

### Animals

The Regional Ethics Committee of Tabriz University of Medical Sciences approved all experimental procedures. Every effort was made to minimize the number of animals used and their suffering. Animals were obtained from the colony of Tabriz University of Medical Sciences. The experiments were performed on adult male wistar rats weighting 220-300g and 3-4 months old at the start of the experiments. The animals were housed in a temperature (23±1°C) and humidity-controlled room. The animals were maintained under a 12:12 h light/ dark cycle, with lights off at 8:00 p.m. Food and water was provided ad libitum except for the periods of behavioral testing. The behavioral testing was done during the light phase.

The experiments were performed on sixty -three rats randomly divided into 9 groups (n=7) as follows:

- Control group: that received intrahippocampal saline (as solvent)
- Lesion group: that received 3 µl intrahippocampal ethidium bromide 0.01%
- Vitamin C groups: that received different doses (0.2, 1, 5 mg/kg) of vitamin C after lesion
- Progesterone groups: that received different doses (0.01, 0.1, 1 µg/µl) of progesterone after lesion
- Combination therapy group: that received vitamin C (5 mg/kg) and progesterone (0.01 µg/µl) after lesion

### Surgery

Before surgery, animals were anesthetized with i.p. injections of ketamine (60 mg/kg body weight) and xylazine (12 mg/kg body weight)<sup>23</sup> and placed on rat stereotaxic instrument in the skull-flat position. Demyelination was induced bilaterally by direct single injection of 3 µl of 0.01% ethidium bromide in sterile 0.9% saline<sup>24</sup> at the rate of 1 µl/min into the DG of hippocampal formation, using appropriate stereotaxic coordinates (AP = -3.8; ML = +2; DV = +3.6).<sup>25</sup> Animals in experimental groups received different concentration of vitamin C (0.2, 1, 5 mg/kg)<sup>26</sup> or progesterone (0.01, 0.1, 1 µg/µl)<sup>19</sup> for 7 days post lesion.

### Step-through passive avoidance apparatus

The apparatus used for PA training and procedure were fundamentally the same as described in our previous studies.<sup>27</sup> It consisted of a two compartment box. An illuminated chamber (30 × 21 × 20 cm<sup>3</sup>) made of transparent plastic was connected by a guillotine door to the dark compartment (30 × 21 × 20 cm<sup>3</sup>) that had black opaque walls and ceiling. The floors of the two compartments were constructed of stainless steel rods (3 mm in diameter, 10 mm apart) through which footshock could be delivered from a constant current source.

### Retention test

After 24 h of PA training, the rat was placed in the illuminated chamber and 10s later the guillotine door was raised and the latency of entering the dark compartment (STL: step-through latency) was recorded during 10 mins.

### Analysis

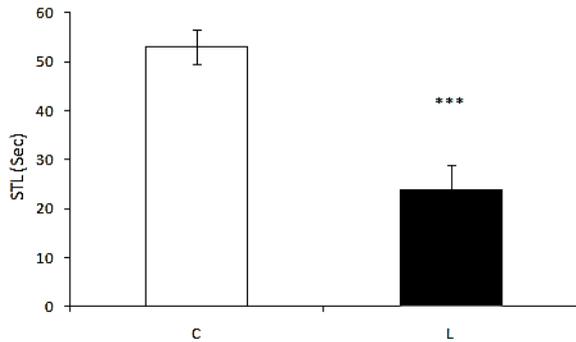
Data are expressed, as means ± SEM. The statistical analysis of the data (latency 24 h after training) was carried out by one-way ANOVA-followed by Tukey's test. In all comparisons, P < 0.05 was considered significant.

### Results

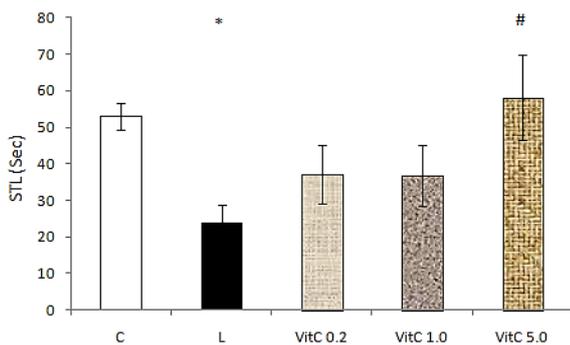
As shown in Figure 1, an independent t-test indicated that there is a significant difference (F=1.520, p<0.001) in STL between the control (53.38±12.64) and lesion (23.71±1.88) groups. This result suggests that injection of ethidium bromide in DG of hippocampus effectively reduces STL and deteriorates passive avoidance learning. A one-way ANOVA comparison between groups also showed that there is a significant difference (F= 3.946, p<0.05) between lesion group (23.71±1.88) and the group that received 5 mg/kg vitamin C intrahippocampally (58.14±27.96) (Figure 2).

Statistical analysis of STL in progesterone groups (which is shown in Figure 3) showed that induction of lesion with ethidium bromide in the hippocampus and injection of high dose of progesterone (1 µg/µl) significantly (F=3.391, p<0.05) decreased passive avoidance learning. As illustrated in Figure 4, a one-way ANOVA revealed that the STL in combination therapy group (0.01 µg/µl of

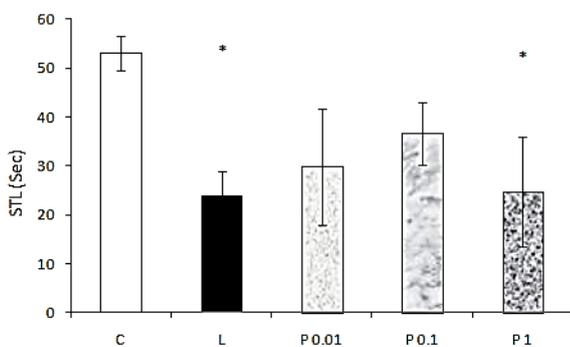
progesterone + 5 mg/kg of vitamin C) was significantly ( $F=5.691$ ,  $p<0.05$ ) more than the lesion group ( $23.71\pm 1.88$ ) and combination therapy could promote passive avoidance learning. However, there was no difference between the effective dose of vitamin C and combination group (Figure 4). These results together showed that progesterone could not potentiate the effect of vitamin C on memory.



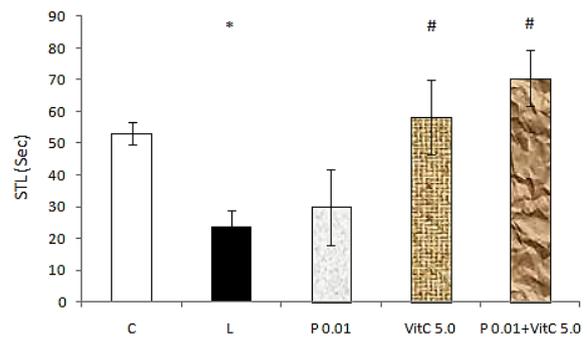
**Figure 1.** The effect of pretraining intrahippocampal injection of ethidium bromide on STL. C: control, L: lesion. \*\*\* $p<0.001$  when compared with control group. Data are expressed as mean  $\pm$  SEM for  $n = 7$  animals per group.



**Figure 2.** The effect of pretraining intrahippocampal injection of different doses of vitamin C on STL. C: control, L: lesion, Vit C: vitamin C with doses of 0.2, 1.0 and 5.0 mg/kg. \* $p<0.05$  when compared with control group and # $p<0.05$  when compared with lesion group. Data are expressed as mean  $\pm$  SEM for  $n = 7$  animals per group.



**Figure 3.** The effect of pretraining intrahippocampal injection of different doses of progesterone on STL. C: control, L: lesion, P: progesterone with doses of 0.01, 0.1 and 1.0  $\mu\text{g}/\mu\text{l}$ . \* $p<0.05$  when compared with control group. Data are expressed as mean  $\pm$  SEM for  $n = 7$  animals per group.



**Figure 4.** The effect of pretraining intrahippocampal injection of vitamin C (5mg/kg) plus progesterone (0.01  $\mu\text{g}/\mu\text{l}$ ) on STL. C: control, L: lesion, P 0.01: progesterone 0.01  $\mu\text{g}/\mu\text{l}$ , Vit C vitamin C 5 mg/kg. \* $p<0.05$  when compared with control group and # $p<0.05$  when compared with lesion group. Data are expressed as mean  $\pm$  SEM for  $n = 7$  animals per group.

### Discussion

In the present study, the effect of vitamin C and progesterone was evaluated on memory in MS. Their effect was assessed on a passive avoidance task through the intrahippocampal administration in male wistar rats. In order to induce MS, direct injection of ethidium bromide in DG region of hippocampus was performed and then the effect of vitamin C and progesterone on passive avoidance memory investigated. Vitamin C had a promoting effect on memory task and increased STL in comparison with the vehicle group. Progesterone in lower doses had no effect on STL, but in a high dose significantly decreased PAL. This experience does not appear to be due to a nonspecific effect of intrahippocampal injection, while saline injection had no effect on the learning capability of rats.

Intrahippocampal microinjection of vitamin C (5 mg/kg in) in our study increased STL significantly and our results are compatible with the results of other studies.<sup>15,28-31</sup> A study of the oral supplementation of vitamin C indicated that it could attenuate the risk of dementia in aged mice.<sup>28</sup> Shahidi et al. (2008) showed that i.p. administration of vitamin C could improve acquisition and retention in passive avoidance process in intact rats.<sup>15</sup> Two different studies also showed that i.p. injection of vitamin C could be useful in retention of memory in the scopolamine treated rats<sup>29</sup> and impede amnesia in homocysteine administered rats.<sup>30</sup>

One of the possible mechanisms of vitamin C on learning and memory is the modulatory role on neurotransmitter systems such as cholinergic<sup>14,29</sup> and serotonergic<sup>29</sup> systems. In addition, vitamin C restores acetyl cholinesterase activity, which has an essential role in learning and memory processes.<sup>31</sup>

Hippocampus has an important role in learning and memory processes<sup>32</sup> and oxidative stress is considered to be a probable cause due to impairment in hippocampal function.<sup>10</sup> Vitamin C is an antioxidant<sup>14,33,34</sup> that scavenges the ROS and prevents lipid per oxidation;<sup>8</sup> therefore, intrahippocampal injection of vitamin C probably has mediated its function through one or some of these mechanisms in this study.

Intrahippocampal administration of progesterone in this study had different effects on passive avoidance learning and memory. Progesterone has different effects in memory including enhancement, no effect or decrease of memory formation.<sup>35,36</sup> 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.<sup>37</sup> On the other hand, some studies demonstrated that subcutaneous injection of progesterone impairs social recognition memory,<sup>18</sup> spatial working and reference memory performance, cognition function and avoidance tasks in young rats and mice,<sup>38</sup> but it does not impair novel object recognition task.<sup>36</sup> Our results are consistent with El-Bakri et al. (2004) study.

Progesterone has different mechanisms depending on the type of task. Intraperitoneal injection of progesterone in mice can impair retention of memory through facilitation and reinforcement of GABAergic activity, which attenuates arousal levels.<sup>35,39</sup> Some studies indicated that reduction of neuronal activity through activation of GABA receptors by progesterone may produce analgesic or anxiolytic that undermines cognitive function.<sup>19,36</sup> It is also important to consider that as males have higher levels of steroid receptor co-activators, which enhance steroid hormone action in many brain regions,<sup>40</sup> it is likely that lower levels of progesterone are sufficient to elicit a physiological response within the male brain.<sup>18</sup> These data are consistent with that in female mice that have lower plasma levels of progesterone in diestrus, which helps them to learn to avoid footshock faster than females in estrus.<sup>39</sup> In our study dose, 1 µg/µl, progesterone deteriorated the passive avoidance learning that is consistent with the findings of Bychowski 2012 and Bimonte-Nelson 2004. Given the observed different effects of progesterone, it indicates that various factors affected the results such as: model of administration, behavioral test kind, gender, age of animal, time of hormone treatment, and dose of hormone.

### Conclusion

In conclusion, this study showed that intrahippocampal injection of vitamin C (5 mg/kg) improves passive avoidance learning in ethidium bromide model of MS, whereas progesterone alone or in combination with vitamin C does not improve or affect the memory.

### Acknowledgments

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### Ethical Issues

The study protocol was designed in accordance with NIH guidelines and Ethics Committee for the Use of

Animals in Research at Tabriz University of Medical Sciences.

### Conflict of Interest

The authors report no conflicts of interest.

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