

Learning and Memory Impairment Induced by the Injection of Ascorbic Acid and Ascorbate Oxidase into the Hippocampus in the Morris Water Maze

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ABSTRACT

BACKGROUND AND OBJECTIVE: Ascorbic acid has a wide range of functions in the central nervous system such as neuromodulator and antioxidant. Ascorbic acid intervenes with the neurotransmitters involved in learning and memory. In this study, we examined the effects of its injection and its removal enzyme in the hippocampal CA1 region on spatial learning and retention.

METHODS: We used 49 Wistar rats in this study (220-270 mg/kg) and we divided them into seven groups including: control, sham (recipients of solvent), ascorbate oxidase (0.2, 0.4 µg/kg), ascorbic acid (24, 12µg/kg), and inactive ascorbate oxidase (0.2 µg/kg). Bilateral cannula was performed in the hippocampal CA1 region using stereotaxy device. After one week of recovery, one microliter of the drugs was injected by a Hamilton syringe. Spatial learning and retention was measured by using the Morris water maze.

FINDINGS: The results show that 12 µg/kg dose of ascorbic acid increases mileage (1012.98±63.55) and escape latency (55.48±2.38) compared to the control group (633.33±18.46) (45.9±1.84) (p<0.05). Also mileage (1123.73±108.89) and escape latency delay (57.31±1.18) were raised with 24 µg/kg dose of ascorbic acid compared to the control group (p<0.01). It was determined that ascorbate oxidase with both 0.2µg/kg (p<0.01) and 0.4µg/kg (p<0.001) dose increased the mileage and escape latency compared to the control group.

CONCLUSION: The results showed that the injection of ascorbic acid and its removal enzyme in the hippocampal CA1 region leads to spatial learning and retention loss.

KEY WORDS: Spatial Learning and Retention, The Morris Water Maze, Ascorbate Oxide, Ascorbic Acid, Hippocampus.

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Introduction

Ascorbate acid, besides being an antioxidant, acts as cofactor in many enzymatic reactions, and it has also been recently introduced as neuromodulator in central nervous system (1-3). This vitamin accumulates in the brain of mammals more than other tissues, but it does not synthesize in the brain of mammals. This compound is transferred to brain and neurons by an active transport and diffusion in the choroid plexus absorption mechanism and by SVCT2 which is present in the neuroepithelial cells of choroid system. This active transferring system which is saturable in choroid system allows ascorbate to transfer from plasma to cerebrospinal fluid by a two-stage mechanism, and then to neurons (4).

Ascorbic acid is found in high volumes in striatum, hypothalamus, hippocampus and basal ganglia in the brain (5). Condensation of ascorbic acid in the regions related to memory and learning highlights the role of the mentioned neuromodulator on the process of memory and learning. This vitamin is released from the end of glutamatergic neurons in brain and it can highly regulate the activities of two glutamatergic and dopaminergic systems which are important neurotransmitters in learning and memory processes (6).

Hitherto, there have been several studies on the role of ascorbic acid on the process of memory and learning (7-11). For example, it has been determined that intraperitoneal injection of midterm ascorbic acid reduces spatial learning of rats in radial maze and it is clear that a higher dose of ascorbic acid has more effects on reduction of learning (8). Also it is indicated that long-term oral administration of ascorbic acid to older rats does not disorder spatial learning and retention, but it somewhat improves the working memory, unlike the short-term intraperitoneal injection to younger rats (9). Also it is reported that ascorbate (1 gr/kg i.p.) can significantly reduce avoidance learning level in the shuttle box. Dopaminergic system in corpus striatum of rats plays an important role in avoidance activities, and intraperitoneal injection of ascorbic acid leads to its opposite action (15). Also it has been determined that injection of two 8 and 4 microliters of ascorbic acid into ventral tegmental area of rats accordingly increases and reduces spatial learning in eight-arm radial maze (8). It was reported in another study that injection of ascorbic acid into the cerebral ventricles leads to reduction of spatial learning in rats in Morris water maze (10).

It was reported that positive effects of injecting dopamine D₂ agonist receptor in hippocampus on

memory and learning are reduced by injecting D₂ agonist receptor and ascorbic acid simultaneously (11). CA1 hippocampus is the most important region involved in spatial learning and retention and it is also in one of brain regions that release ascorbic acid, therefore it is a suitable region to study the role of ascorbic acid neuromodulator on nervous structures involved in memory and learning. Considering the high volume of ascorbic acid in brain and the influence that the involved neurotransmitters in memory and leaning and ascorbic acid have on each other, and also because there are some controversies in the results of studies, we compared the effect of injecting ascorbic acid and its removal enzyme with the emphasis on measuring the density of the region on spatial learning and retention in male Wistar rats by injecting ascorbic acid in CA1 region to increase the level of neuromodulator and destructive and reducing ascorbate oxidase of ascorbic acid.

Methods

We studied 49 male wistar rats in this study, which were about 220-270 grams with an age range of 8 to 10 weeks. Rats were kept under 12-hour darkness and 12-hour light cycle in controlled temperature (21±2 centigrade), and they had free access to water and food except during the test. In order to inject in CA1 hippocampus region, stereotaxy surgery was performed by using stereo tax set (Astolting of America). Before performing the surgery, the animal was anesthetized by an intraperitoneal injection of 10% ketamine and 2% xylazine (6 milligrams of ketamine and 4 milligrams of xylazine for each kg of the rat's weight).

After induction of anesthesia and fixing the rat on the stereo tax set, 3.3 mm oral bar were placed below horizontal zero to reach the flat situation of skull in accordance with Atlas of Anatomy. After omitting the surface tissues and finding bregma and lambda regions, bilateral cannula of hippocampus was done with these coordinates: ML=±2.2mm, DV=-3.2mm, AP=-3.8 mm. In order to ensure about the place of injection, a few incisions about 100-150 microns of the position of cannula was provided using Vibroslice device. Then, by the using stained Nissl the target regions were stained and after that they were studied and approved by use of light microscope (fig 1). After a period of recovery (one week after the surgery) the injection of drugs was performed bilaterally in CA1 region by the using 1 microliter Hamilton syringe.



Figure 1. Brain cutting prepared with the cannula region (CA1 hippocampus)

To conduct a behavioral test, the animals were divided into 7 groups including the control group (healthy rats without any surgery), sham group (recipients of solvent), ascorbic acid groups (Darou Pakhsh) (AA) (24, 12 $\mu\text{g}/\text{kg}$), ascorbate oxidase groups (Sigma-Aldrich) (AO) (0.2, 0.4 $\mu\text{g}/\text{kg}$), and inactive ascorbate oxidase groups (Sigma-Aldrich) (I-) AO (0.2 $\mu\text{g}/\text{kg}$). The ascorbic acid was injected 30 minutes before the test and active and inactive ascorbate oxidase were used 20 minutes before the test with the speed of 1 micro liter per minute. The sham group received physiological serum as a solvent.

In this study we used Morris water maze to evaluate spatial learning and retention (12). Morris water maze is a dark color circle shaped pond with a diameter of 136 centimeters and 60 centimeters height and about 25 centimeters of this height is filled with 20 ± 2 degrees water. This pond is divided into four equal quadrants and there is one specific point of each quarter for the animal to be released into the water. A hidden metal platform with the diameter of 10 centimeters was fixed in the center of southern quadrant about 1.5 centimeters under the water. The circle was used as a target quadrant. The one-day protocol was used in this study in order to evaluate spatial learning and retention. The acquisition test is consisted of three blocks which are performed 30 minutes apart, and each block has four trials. In each trial, the animal is released into the water through one of the four randomly chosen quadrants and it has approximately 60 seconds to find the hidden platform under the water by observing the surrounding clues and rest on it.

After being placed on the platform, the animal rests on it for 30 seconds. In each block, the animal is released into the water from 4 different quadrants. In this test, escape latency and mileage to the hidden platform in these three blocks are criteria for calculating spatial learning. Two hours after the last trial, the Probe

test which is for evaluating the retention of animals was performed. This test (forth block) includes an individual trial in which the hidden platform is removed from the maze and the animal is released into the water through the opposite quadrant of the target quadrant and has 60 seconds to swim freely in the water. The evaluating criteria in this test are the time of presence and mileage in the target quadrant. Images were taken by a fixed camera on the upper part of the central pond with a speed of 25 images per second and were received by a monitor. After that, they were recorded and analyzed by maze router. At the end of the behavioral test, the density of ascorbic acid in rat's hippocampus was measured by applying the Roe and Kuether method (13). In this method rats were anesthetized with CO_2 gas, and the animal's head was separated by a guillotine and its hippocampus was frozen into liquid nitrogen after the segregation. Then, each hippocampus was weighted and about 9 times of its volume was added as buffer (Perchloric acid 0.35 M with 0.1 mg/ml EDTA), and they were homogenized in zero Celsius degree. The samples were centrifuged with 15000 rpm speed and for three minutes in 4 centigrade degrees. 200 μL of the supernatant was poured into a micro tube, and 50 μL identifier was added and then the samples were incubated for three hours in 37 centigrade degrees. Then, the samples were taken into zero Celsius degree and 85% sulfuric acid was added to them. The optimum density was measured in 515 nm length wave, and for calculating ascorbic acid in each sample, the obtained value was put into standard curve formula. In order to compare the tested groups, ANOVA test and subsequently the Tukey test were taken and $p < 0.05$ was considered significant.

Result

According to the results of the learning section, in the group receiving ascorbic acid with 12 $\mu\text{g}/\text{kg}$ dose, the mileage to the hidden platform was 1012.98 ± 63.55 which, compared to the control group (633.33 ± 18.46), shows an increase ($p < 0.05$). Also, in the group receiving ascorbic acid with 24 $\mu\text{g}/\text{kg}$ dose, the mean of mileage to the hidden platform was 1123.73 ± 108.89 which, compared to the control group (633.33 ± 18.46), shows increase ($p < 0.01$) (fig 2A). The mean of mileage in groups receiving ascorbic acid with 24, 12 $\mu\text{g}/\text{kg}$ dose were 55.48 ± 2.38 and (57.31 ± 1.18) accordingly which compared to the control group 45.9 ± 1.84 shows an increase ($p < 0.01$) (fig 2B).

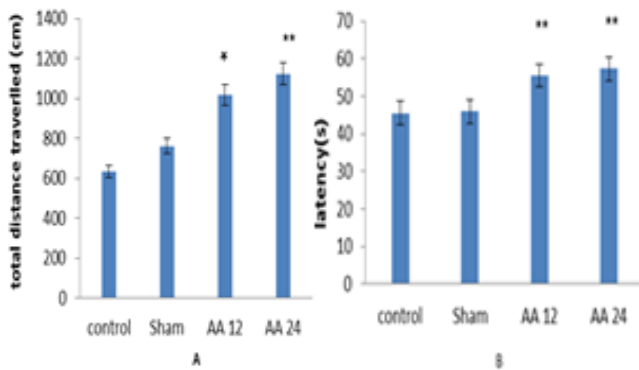


Figure 2. A) comparison of mileage to the hidden platform in the mean of three blocks. B) comparison of escape latency in the mean of three blocks

Ascorbic acid (AA), control, sham (n=7).

*p<0.05 and **p<0.01 in comparison with control and sham

The results of evaluating retention by a Probe test showed that intra-hippocampal injection of ascorbic acid with a 12 µg/kg dose leads to a reduction in mileage in the target quadrant (45.10±2.91) when compared to control group (26.19±1.71) (p<0.05). Injection of ascorbic acid with a 24 µg/kg dose also leads to a reduction in the mean of mileage in the target quadrant (27.11±0.95) when compared to the control group (p<0.01) (fig 3A). Also it was determined that the percentage of time spent in the target quadrant in groups receiving ascorbic acid with 24. 12 µg/kg dose are 17.11±2.29 and 16.33±0.49 accordingly, which shows reduction when compared to the control group (20.95±2.41) (p<0.05) (fig 3B).

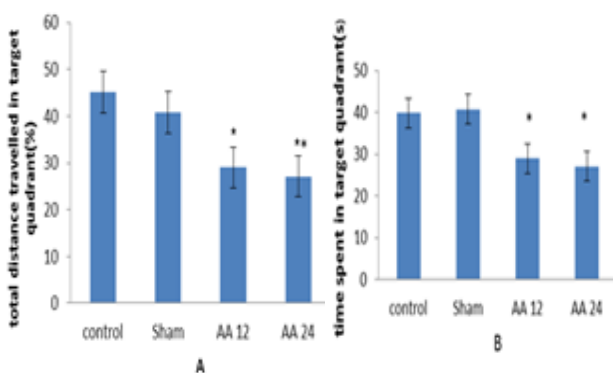


Figure 3. A) comparison of the mileage percentage in the target quadrant in the fourth block. B) comparison of the time spent mean in the target quadrant in the fourth block

Ascorbic acid (AA), control, sham (n=7).

*p<0.05 and **p<0.01 in comparison with control and sham

Evaluating the effects of hippocampal injection of ascorbate oxidase on the mean of mileage to the hidden

platform in 0.2 µg/kg dose of ascorbic acid is 1251.58±92. 74, which shows a significant difference compared with the control group (760.62±101.43) (p<0.01) (fig 4A).

The mileage to the hidden platform in the group receiving ascorbic acid with a 0.4 µg/kg dose is 1263.79±81.78, which shows a significant difference compared with the control group (p<0.001). Also, the mileage to the hidden platform in both 0.4, 0.2 µg/kg doses of ascorbic acid showed increase when compared to inactive ascorbic acid group (831.17±51.38) (p<0.01) (fig 4A).

Escape latency in groups receiving 0.4, 0.2 µg/kg doses of ascorbic acid are 56.16±1.96 and 57.8±1.3 accordingly, which shows increase when compared to the control group (45.89±0.71) (p<0.001). The mean of escape latency in 0.2 µg/kg (p<0.01) and 0.4 µg/kg (p<0.001) doses of ascorbic acid also increased compared to inactive ascorbic acid group (47.53±1.01) (fig 4B).

The results of intrahippocampal injection of ascorbic acid on the mileage percentage in the target quadrant in the Probe test showed that ascorbic acid in both 0.2 µg/kg (p<0.05) and 0.4 µg/kg (p<0.01) doses lead to a reduction in mileage when compared to the control group (fig 5A). Also, in 0.2 µg/kg (p<0.05) and 4 µg/kg (p<0.01) doses of ascorbic acid lead to a reduction in escape latency in the target quadrant when compared to the control group (fig 5B).

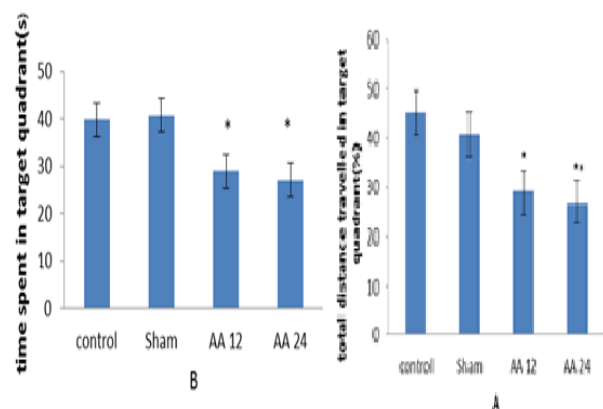


Figure 4. A) comparison of the mileage mean to the hidden platform in the mean of three blocks. B) comparison of the escape latency mean in the mean of three blocks

Ascorbate oxidase (AO), inactive ascorbic acid (I-AO), control, sham (n=7).

p<0.01, *p<0.001 in comparison with control and sham

###p<0.01, ##p<0.01 in comparison with I-AO

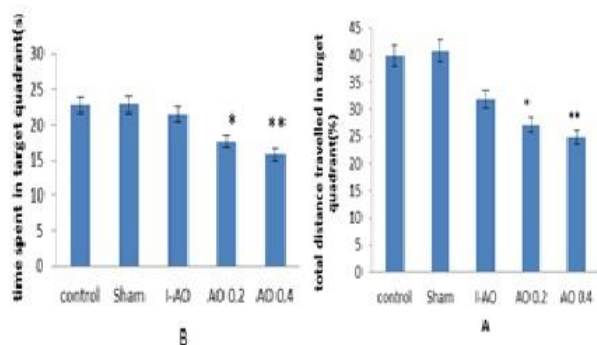


Figure 5. A) comparison of the mileage percentage in the target quadrant in the fourth block. B) comparison of the time spent mean in the target quadrant in the fourth block

Ascorbate oxidase (AO), inactive ascorbic acid (I-AO), control, sham (n=7).

*p<0.05 and **p<0.01 in comparison with control and sham

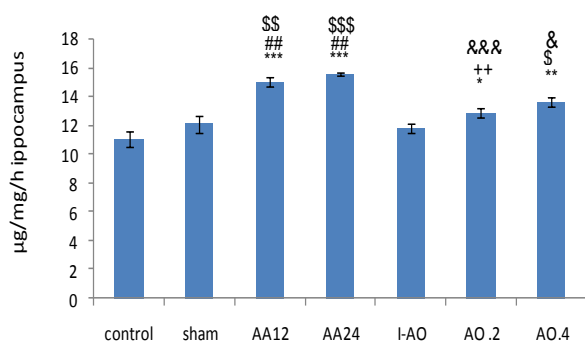


Figure 6. Comparison of ascorbic acid density in hippocampus of tested groups including control, sham, AA, AO, I-AO (n=7).

*p<0.05, **p<0.01, ***p<0.001 in comparison with control

^{##}p<0.001 in comparison with sham and control

[§]p<0.05 and ^{\$\$\$}p<0.001 in comparison with I-AO

⁺⁺p<0.01 in comparison with AA12

[&]p<0.05 and ^{&&&}p<0.001 in comparison with AA24

Discussion

We studied the effects of injecting ascorbic acid and ascorbate oxidase in CA1 hippocampus region of male wistar rats on spatial learning and retention by Morris water maze, and the results showed learning and memory impairment after injection of both above compounds into the hippocampus.

According to the results of the present research it was found that the injection of ascorbic acid increases the mileage and escape latency to the hidden platform in the mean of three blocks, which confirms the reduction of spatial learning. Evaluating the results of Probe test which shows some changes in the memory,

indicates that injection of ascorbic acid (24 µg/kg) into the hippocampus decreases mileage in the target quadrant of the fourth block. Also the escape latency percentage decreases in the target quadrant of the mentioned block after the injection of ascorbic acid which shows the reduction of retention.

Destructive effects of peripheral and central injection of ascorbic acid on spatial learning and retention has been reported in the previous studies (8 and 10). One of the logical reasons for destruction of spatial learning and retention after the injection of ascorbic acid is the dependence of this vitamin to dopaminergic system. In the literature, this drug is accompanied with inhibition of the dopaminergic system and it reduces the binding of dopamine agonist receptors (D₁ and D₂) and causes destruction in learning and retention by intervening with the function of dopamine (14-16). D₂ dopamine agonist receptors affect the pre-synaptic terminals (17).

It is determined that ascorbate has functions similar to D₂ dopamine agonist receptors (17). In this regard researches show that injecting D₂ dopamine agonist receptors in CA1 region leads to improvements in spatial learning and retention of rats, and this effect decreases with co-administration of ascorbic acid and D₂ dopamine agonist receptors (11). Therefore, it is possible that after the injection, ascorbic acid intervenes with presynaptic dopaminergic receptors and reduces learning and retention. Also studies show that hippocampus receives dopaminergic inputs from VTA region. These mentioned dopaminergic neurons affect pyramidal cells of CA1 hippocampus region by affecting the D₁/D₅ receptors and have effect on creating long-term memory (18). Therefore, considering the role of ascorbic acid dopaminergic agents (17), it is possible that this vitamin cause impairment in memory and learning by affecting the dopamine receptors.

As was mentioned before, ascorbic acid is secreted from the glutamatergic neurons in brain and highly regulates the activities of glutamatergic system. It is determined that ascorbate acts as NMDA antagonist receptor in high doses (100-500 mg/kg) and probably ascorbate decreases the release of dopamine by antagonizing the NMDA receptors which is followed by a reduction in spatial learning and retention (19). Since most of the aggregation of NMDA receptors are in the CA1 region and dentate gyros of the hippocampus, it is safe to say that ascorbic acid increases retention by inhibiting these receptors (19). Another mechanism about the development of inhibitory effects of ascorbic

acid on learning and memory parameters is blocking the L-type calcium channels with ascorbic acid. It is reported that pharmacological blockage of the L-type calcium channels can impair learning (20).

Considering the fact that ascorbic acid has antioxidant activities and it has been shown that the effects of these compounds on improving retention and learning is because of its antioxidant feature (7 and 8). Therefore, there are some contradictions between the results of this study and potentiating effect of antioxidant compounds on learning and memory. Since ascorbic acid was introduced as a neuromodulator in the central nervous system (21) and the intervening effect on the function of other neurotransmitters is an especial effect, while the antioxidant effect is a general effect. The intervening effect of ascorbic acid on the neurotransmitters such as dopamine, glutamate and serotonin is more than its antioxidant effect on learning, it means that ascorbic acid impair spatial learning and retention by inhibiting NMDA and calcium channels (21). In the present study, injecting two doses of ascorbate oxidase (0.4, 0.2 $\mu\text{g}/\text{kg}$) caused a significant increase in two parameters of mileage and escape latency in the mean of three blocks.

Also in the Probe test for evaluating retention, it was determined that injecting both doses of ascorbate oxidase in CA1 hippocampus region decreases the mileage percentage in the target quadrant and escape latency percentage in the target quadrant, which shows the reduction of spatial learning and detention. There was no significant change in parameters after the injection of inactive ascorbate oxidase. Therefore, we cannot relate the impairments caused by injecting ascorbate oxidase to the existence of this glycoprotein with a high molecule weight. So the reason for learning and detention decrease is related to the enzymatic activities of this compound which can eliminate ascorbic acid in the injection region which is CA1 of the hippocampus. The literature review shows 50-70 percent omission of ascorbic acid extracellular striatal 20 minutes after the injection of ascorbic acid in this region (22).

There is this possibility in this study that ascorbate oxidase inhibited the dopamine receptors and/or dopamine molecules by binding to them and applied its decreasing effect on them by this way, too. In fact, when

considering the ascorbic acid's functions such as being neuromodulator, neurotransmitter, antioxidative stress or enzyme, it is obvious that ascorbate oxidase can intervene with each of these functions. Measuring the level of hippocampus ascorbic acid level showed that the density of ascorbic acid in the groups which received ascorbic acid (12, 24 $\mu\text{g}/\text{kg}$) decreased significantly when compared to the control group, but in the groups that received ascorbate oxidase (0.2, 0.4 $\mu\text{g}/\text{kg}$) there was a significant increase in ascorbic acid in hippocampus when compared to the control group, but this increase in density is not in accordance with behavioral results. In the previous studies, we also witnessed that injection of ascorbate oxidase in dorsal hippocampus did not have an effect on behaviors, but in the striatum the extracellular ascorbate is decreased and the behaviors are dysfunctional (18).

In this study it is possible that the reason for the increase in ascorbic acid density is oxidative stress which is due to a tissue inflammation that is caused by making a cannula in the place which leads to a higher aggregation of ascorbic acid in the region to act as an anti-stress agent (23). The other reason for increase in ascorbic acid density can be related to the activities of ascorbic acid carriers. The activities of these carriers are subordinate to density and abundance of ascorbic acid. In fact, elimination of ascorbic acid can lead to stronger groupings and more mobilized intracellular resources and also carriers increasing the level of ascorbic acid in extracellular space (4). Overall, the results of this study showed that an increase in the level of ascorbic acid and decrease of this neuromodulator's level in CA1 region, both destroy detention. Therefore, considering the various roles of ascorbic acid, it seems that there is only one specific concentration range for spectral function. Outside of that zone, spatial learning and detention of animals would fall in trouble by intervening with the neurotransmitter function or a change in enzymatic and antioxidant functions of this vitamin.

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