

## Effects of Aluminum chloride (AlCl<sub>3</sub>) on spatial memory: association with oxidative stress

Elaheh Asili <sup>1</sup>, Mohammad Sharifzadeh <sup>2\*</sup>

1. Faculty of Pharmacy, Islamic Azad University-Pharmaceutical Sciences Branch (IAUPS), Tehran, Iran

2. Dept. of Pharmacology and Toxicology, School of Pharmacy, Tehran University of Medical Sciences (TUMS), Tehran, Iran

### ABSTRACT

The effects of chronic AlCl<sub>3</sub> exposure on spatial memory in rats remain controversial. Since some toxic metals such as aluminum when entered the body, can contribute to diseases such as Alzheimer, in this study the time course effect of the systematically administered AlCl<sub>3</sub> on the spatial memory retention in the Morris Water Maze was investigated. Rats were treated with AlCl<sub>3</sub> (1 gr/lit) for two, four and eight week periods. Rats in all groups were trained for four days, each day included one block, and each block contained four trials. Test trials were conducted 48 hours after the completion of AlCl<sub>3</sub> treatment. The results of our study show that AlCl<sub>3</sub> increased the escape latency and the traveled distance by the AlCl<sub>3</sub> treated rats in comparison to the controls, but no significant difference in the swimming speed between the two groups was observed. This findings suggests that AlCl<sub>3</sub> has caused significant spatial memory retention impairment in the groups that received it for a period of eight weeks. Also the levels of plasma thiol groups and plasma antioxidant capacity showed a significant decrease in rats given AlCl<sub>3</sub> for four and eight weeks in comparison with the control group. Our results also show that neurotoxicity which is caused by aluminum is associated with oxidative stress. Oxidative damage leads to formation of amyloid plaques in the nerve cells which in turn leads to decrease in the learning capability of the lab animals. Therefore, the results of our study provide a recommendation to people chronically exposed to Al, to consume fresh vegetables and fruits and/or take safe doses of antioxidant supplements to strengthen their defense mechanism against the antioxidants.

**Key words:** Spatial Memory Retention, aluminum chloride, oxidative stress, Alzheimer, Morris Water Maze, neurotoxicity, antioxidant

\*Corresponding Author : Dr.Mohammad Sharifzadeh Dept. of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences (TUMS), Tehran, Iran. P.O.Box: 6451-14155  
Tel: +98 21 66482705 Fax: +98 21 66482705  
e-mail: msharifzadeh@sina.tums.ac.ir

## 1. Introduction

Metals such as aluminum, lead, mercury and nickel enter the body through food and air. The toxic substances generated from these metals remain in the body for several years and contribute to illnesses such as Alzheimer, Al poisoning and other diseases (Crapperm *et al.*, 1973). Patients on dialysis (Alfrey *et al.*, 1976) or on long-term treatment with total parenteral nutrition (Klein, 1993) have been shown to accumulate aluminum in different organs. The toxicological effects of Al accumulation in the body include encephalopathy (Alfrey *et al.*, 1976), bone disease (Ward *et al.*, 1978) and anemia (Short *et al.*, 1980). Al is also described as a possible contributor to Alzheimer disease (AD) (Campbell, 2002). Epidemiological studies have indicated presence of an association between Al in drinking water and AD. Also a variety of studies on animal and human subjects has implicitly shown a link between memory and learning deficits after exposure to Al (Yokel, 2000; Exley, 2005).

It has been stated that the use of Al as an experimental neurodegenerative agent has recapitulated every sign of degenerative spiral which afflicts AD patients (Grant *et al.*, 2002). Moreover, additional evidence suggests that since brain cells do not get reproduced unlike the cells of the other organs in the body, toxic substances such as aluminum accumulate in these cells along with aging, resulting in a consequent metabolic malfunctions (Nunomura *et al.*, 2001). Neurotoxicity from exposure to Al is known to result in impairment of learning memory and cognition function both from clinical observations and from animal experiments (Berlyne *et al.*, 1972; Ashall, 1994). Since the exact mechanism of retardation of the brain function due to Al is not yet clearly known (Savory, 2003), and also considering its effect on learning and spatial memory, this study was conducted to assess the neurotoxicity of AlCl<sub>3</sub> and its possible effect on behavior and Spatial Memory Retention, after exposing mice to it chronically by using the Morris Water Maze method.

## 2. Materials and Methods

**Animal treatment:** this study was conducted on a total of 60 adult male Albino Wistar, weighing 200-250 grams. These animals were procured from the animal housing facility of the University of Tehran, and were kept in cages made out of polycarbonate in groups of 5. The temperature of the holding area was kept at a constant temperature of  $23 \pm 2$  °C and photoperiod of 12h light/dark cycle. The solution of AlCl<sub>3</sub> was prepared by dissolving 1 gram of AlCl<sub>3</sub>.6H<sub>2</sub>O salt, weighed by a digital scale, in one liter of tap water. The room which housed the mice was lightened with two 100 V

common light bulbs. Enough water and food was provided for the mice. The water dish for the test group contained the solution of AlCl<sub>3</sub>, and the water dish for the control group contained tap water. For the ease of identification, the tails of the mice were marked using a magic marker.

**Location of the experiments:** the experiments were conducted at the animal housing facility of the department of pharmacological sciences of the University of Tehran.

**Morris Water Maze (MWM):** in this study, the maze, which is widely used for investigate of learning and spatial memory in mice, consisted of a black cylindrical pool (136 cm in diameter and 60 cm in height). The pool was filled to the depth of 25 cm with water  $\pm 2$  °C. An escape platform (10 cm) made of clear Plexiglas was submerged 1.5 cm under the water level. The animal had to swim until it found the hidden platform. The mice generally use signs and cues outside the maze to develop a spatial map of the environment. The pool was divided into four arbitrary quadrants labeled *trials* 1,2,3,4. The platform was located between the trial points 2 and 3. This platform was hidden from the sight of the animal and it was placed in the same spatial location of the pool throughout the entire experiment period. To provide the animals with the spatial cues outside the pool, three visual signs were placed on the wall of the room which guided the mice to the location of the hidden platform. Each mouse was trained for four days; each day, included one block, and each block included four trials. During each trial, the animal was released in the pool at one of the four quadrants of the pool while facing the pool wall. The release point of the animal was decided randomly.

Each animal was given 90 seconds to swim and to find the platform. If the animal succeeded in finding the platform in less than 90 s, it was permitted to rest on the platform for 30 s (Rest Time). The animals that failed to find the platform within 90 s were put in the time out (were gently moved to the platform for 30 s). By doing this the animals were conditioned to know that the only way to escape the water was getting on the platform. The rest time between each trial in each block was 30 s.

The movements of each animal in the maze were recorded by a camera which was connected to a computer. The recorded pictures were later analyzed the Maze Inspector Software.

**Evaluation factors:** three important factors made up the criteria for evaluation of the animal's performance including the time took the animal to find the platform, the traveled distance during each trial and the swimming speed of the animal. This trial was carried out in a special room in which the visible signs such as computer monitor and three other signs were placed on the wall. The animal could find the platform using these visual cues. In the first day of the training trial, the animal traveled long distance and spent more

time to find the platform, but in the second and third days the traveled distance and time were minimized. By the fourth day the animals had learned the location of the platform and had it memorized. All the movements of the animals were sent by a camera to a computer which was programmed specifically for this experiment and the pictures was recorded. The obtain information included the time spent by the animal to find the platform, the time spent in each quadrant, the total distance traveled, the percentage of the distance traveled in each quadrant, the percentage of the entry into each quadrant, the swimming speed and the angle with which the animal started its swimming.

**Evaluation of the effect of the  $AlCl_3$ :** to study and evaluate the effects of  $AlCl_3$  on the spatial memory retention of the animal, three sets of data was collected including escape latency (the time which took the animal to find the platform), traveled distance and the swimming speed. These data were gathered for both the test and the control group. Forty eight hours after the end of the training period, on the sixth day, the spatial memory retention tests were carried out in the MVW, and then blood samples were collected directly from the hearts of the animals.

**Sampling and grouping the animals:** the mice were randomly divided into six groups of ten, three of which were designated as the control group and the rest were labeled as the test group. All the conditions were the same for both groups with the exception of their drinking water. The animals in the test groups were given water which contained  $AlCl_3$ , but the animals in the control groups were given standard tap water. The animals in the first group were given water containing  $AlCl_3$  for a period of two weeks. The animals in the second group were kept under the same conditions with the only exception of receiving standard tap water for two weeks. The animals in third and fourth groups also were kept under the same conditions with the exception of the former receiving  $AlCl_3$ -containing drinking water for a period of four weeks. The animals in fifth and sixth groups were also kept under the same conditions with the exception of the former receiving  $AlCl_3$ -containing drinking water for the entire duration of the experiment.

**Preparation of the samples for blood tests:** first, the animals were placed under anesthesia using ether and were laid on their backs and pinned to the table so that they could not move, and their chests were opened up. Using a syringe, 5 cc of blood was drawn directly from their hearts. Then, the blood was transferred to the test tubes which contained 1 ml of Heparin. The blood plasma samples were separated by centrifuge at the speed of 30000 cycles/s for 10 minutes and stored in closed lid micro tubes in  $-20\text{ }^\circ\text{C}$  so that they could be used later.

**Evaluation of Oxidative Stress level:** to an empty test tube, 1.5 ml of FRAP indicator was added and stored for five minutes in  $37\text{ }^\circ\text{C}$ . Then,  $50\text{ }\mu\text{l}$  of plasma sample was added to the content of this test tube and was stored for ten minutes in  $37\text{ }^\circ\text{C}$ . The next step was measuring the color intensity of the sample with a spectrophotometer at the wave length of 593 nm against the blank (1.5 ml of FRAP indicator+ $50\text{ }\mu\text{l}$  of  $H_2O$ ). The FRAP method is based on the capability of plasma to reduce  $Fe^{+3}$  to  $Fe^{+2}$ .

**Evaluation of the oxidation level of the proteins:** to a test tube containing 1 ml of Tris buffer,  $50\text{ }\mu\text{l}$  of plasma was added and its light absorption capability was measured at the wave-length of 412 nm against the blank (1 ml of Tris buffer), (A1). Then, to these test tubes  $20\text{ }\mu\text{l}$  of DTNB indicator was added, and after 15 minutes being kept in room temperature, its light absorption capability was measured using a spectrophotometer (A2). A1 and A2 values were plugged into the following mathematical formula, and the level of thiol groups was calculated.

$$(A2-A1)*(1.07/0.05)/13.6= (A2-A1)*1.57\text{mM}$$

**Ethical considerations:** throughout the entire experiment, all of the animals were treated ethically and according to the Animal Rights Doctrine.

**Statistical Analyses:** the GraphPad Prism 4.0 software was used to analyze the data. The statistical significance of the differences between the groups was assessed with an analysis of variance (one-way-ANOVA). Where the significant differences in the behavioral data was observed, Newman-Keuls multiple comparison test for post hoc analysis was used, and where the significant differences in oxidative evaluations was observed, Tukey comparison test was used. Statistical significance was assessed at an error level of 0.05 (P-value < 0.05).

### 3. Results

**The effect of training on the escape latency, distance traveled and the swimming speed:** as shown in Fig. 1, there is a significant difference (P-value < 0.001) between the mean of the values for the escape latency and traveled distance in all of the animals in the fourth day of the training in comparison with the first day, but for the swimming speed no significant difference is observed (P-value > 0.05) which indicates the lack of any effect by  $AlCl_3$  on the animals' locomotor activity.

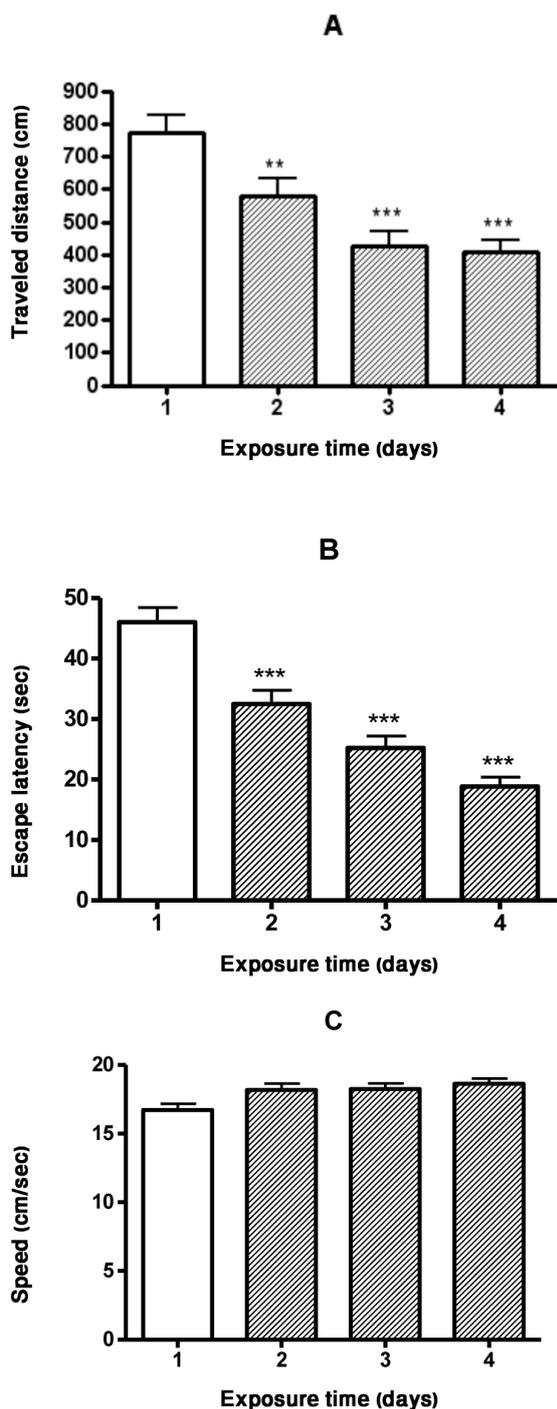


Figure 1. Daily comparison of the traveled distance (A), escape latency (B) and speed (C) within the training period.

*The effect of AIC13 on the escape latency in time courses of 2, 4, and 8 weeks:* as demonstrated in Fig. 2, the treatment with AIC13 (1 mg/ml dosage) in time courses of 2, 4, and 8 weeks (during the spatial memory retention test), resulted in significant decrease of the escape latency in the group treated for 8 weeks (P-value < 0.50), but in the groups treated for 2 and 4 weeks, no significant difference was observable (P-value > 0.05).

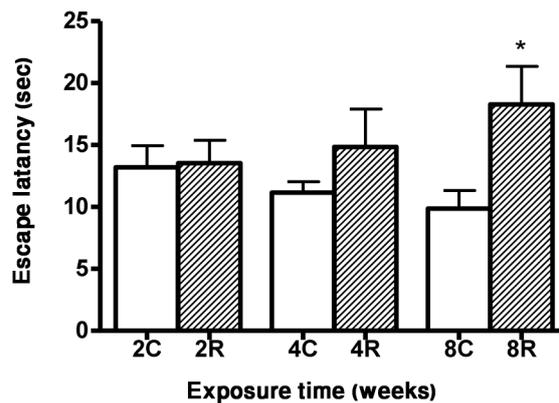


Figure 2. Comparison of escape latency between the AIC13 treated and control groups.

*The effect of AIC13 on the traveled distance in time courses of 2, 4, and 8 weeks:* as demonstrated in Fig. 3, the treatment with AIC13 (1 mg/ml dosage) in time courses of 2, 4, and 8 weeks (during the spatial memory retention test), resulted in significant decrease of the traveled distance in the group treated for 8 weeks (P-value < 0.50), but in the groups treated for 2 and 4 weeks, no significant difference was observable (P-value > 0.05).

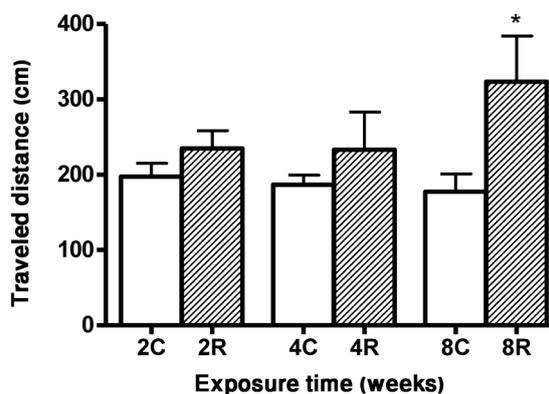


Figure 3. Comparison of the traveled distance between the AIC13 treated and control groups.

**The effect of AlCl<sub>3</sub> on the swimming speed in time courses of 2, 4, and 8 weeks:** as in Fig. 4, the treatment with AlCl<sub>3</sub> (1 mg/ml dosage) in time courses of 2, 4, and 8 weeks (during the spatial memory retention test), resulted in no significant difference in the swimming speed between the test and control groups.

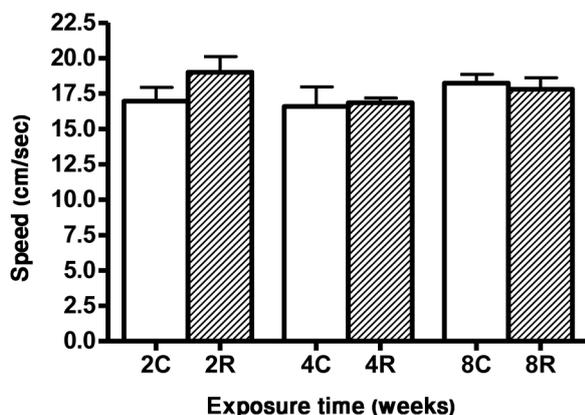


Figure 4. Comparison of the swimming speed between the AlCl<sub>3</sub> treated and control groups.

**The effect of AlCl<sub>3</sub> on the thiol groups of the plasma in time courses of 2, 4, and 8 weeks:** as it can be seen in Fig. 5, the treatment with AlCl<sub>3</sub> (1 mg/ml dosage) in time courses of 2, 4, and 8 weeks (during the spatial memory retention test), resulted in significant difference in the level of thiol groups of the plasma obtained from the test and control groups treated for 4 weeks (P-value < .050) and 8 weeks (P-value < 0.001), but no significant difference was observed between the control and the test group after 2 weeks of treatment with AlCl<sub>3</sub>.

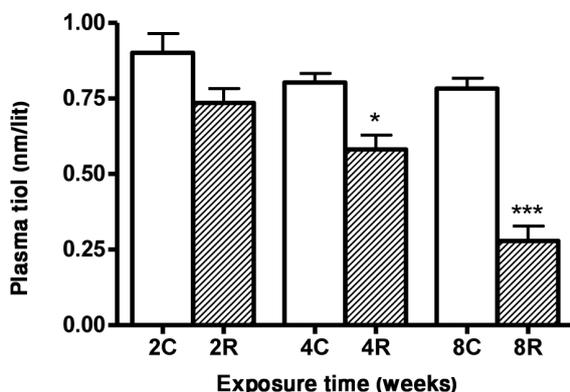


Figure 5. Comparison of the plasma thiol group level between the AlCl<sub>3</sub> treated and control groups.

**The effect of AlCl<sub>3</sub> on the antioxidant activity level of the plasma (FRAP) in time courses of 2, 4, and 8 weeks:** in this test, after the end of the experiment (the end of the sixth day), blood samples were collected from the heart of the animals, and the antioxidant activity level of the plasma was evaluated. As demonstrated in Fig. 6, administration of AlCl<sub>3</sub> for periods of 4 and 8 weeks caused a significant decrease in the antioxidant activity level of the plasma in the two test group in comparison with the animals in the two control groups (P-value < 0.001). Conversely, in the test group which had undergone AlCl<sub>3</sub> treatment for a period of two weeks, no significant decrease in the antioxidant activity level of the plasma was observed in comparison with the control group (P-value > 0.05).

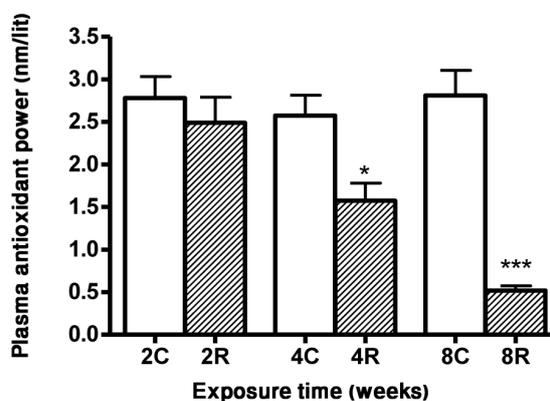


Figure 6. Comparison of the plasma antioxidant power between the AlCl<sub>3</sub> treated and control groups.

**Evaluation of the association between antioxidant activity level of plasma and the escape latency and the traveled distance to the hidden platform:** according to the Fig. 7-A, there is a significant association between the level of antioxidants of the plasma and the escape latency (P-value < 0.05 and R<sup>2</sup> = 0.9027), but Fig. 7-B suggests the absence of a significant association between the level of antioxidants of the plasma and the traveled distance (P-value > 0.05 and R<sup>2</sup> = 0.8555).

**Evaluation of the correlation between the time course of AlCl<sub>3</sub> treatment and the level of thiol groups in plasma and antioxidant activity of the plasma:** as shown in the Fig. 8-A, there is a significant association and correlation between the length of the treatment and the level of thiol groups of plasma (P-value < 0.05 and R<sup>2</sup> = 0.9729). Moreover, as demonstrated in the Fig. 8-B, there also exists a significant correlation between the antioxidant activity level of the plasma and the length of the treatment with AlCl<sub>3</sub> (P-value < 0.05 and R<sup>2</sup>=0.9799).

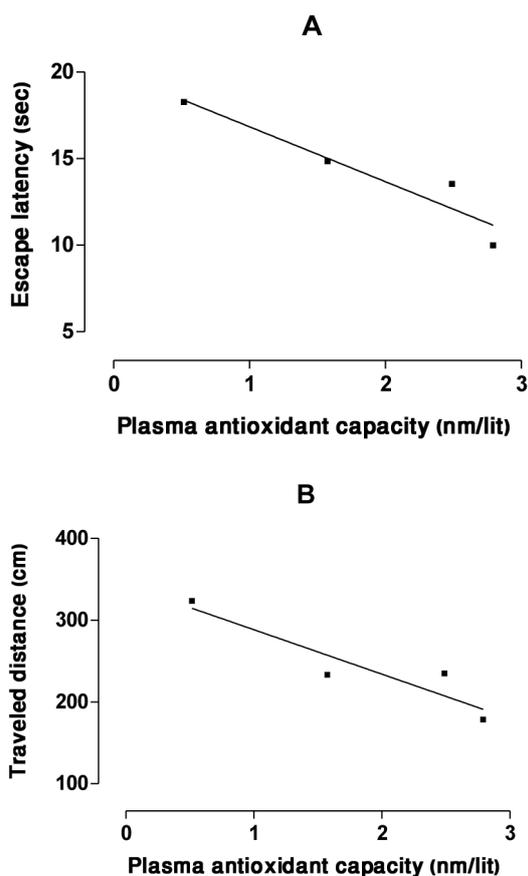


Figure 7. The association between escape latency (A) and the traveled distance (B) to antioxidant activity level of plasma.

#### 4. Discussion

Formation of memory is a sophisticated process requiring different pre-synaptic and post-synaptic precursors (Kuntz *et al.*, 2005). Various studies have been conducted to determine the molecular mechanisms associated with the synaptic changes which contribute to the activities taking place during the development and formation of memory. Many proteins such as transcription factors have been identified (Kuntz *et al.*, 2005). Hippocampus is a part of the limbic system which is needed for formation of various types of memory and learning in mice and the other mammals. In human, any damage to hippocampus leads to disorder in learning capability, memory, remembering places and other undesired mental effects (Bear *et al.*, 1996). Most learning paradigms that require configural associations also require a full functional hippocampus however, learning paradigms that can be solved using only elemental associations can be solved without input from this structure (Morris *et al.*, 1988). Since the Morris Water escape task can test the function of hippocampus

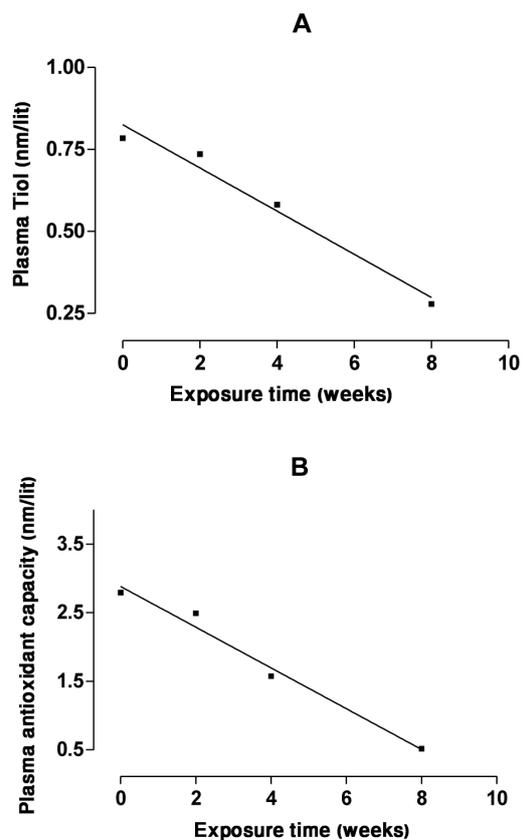


Figure 8. The correlation of the level of thiol groups in plasma (A) and antioxidant activity of the plasma (B) with the time course of AIC13 treatment.

in memory and learning, in animals, familiarity with objects or processes is commonly evaluated through Morris Water Maze which is used to study the spatial memory retention (Kuntz *et al.*, 2005).

In the present study, by using MWM, the effects of AI exposure on the spatial memory of male Albino Wistar mouse in the retention stage was investigated, and the association of AI exposure with oxidative stress parameters were analyzed. Results of the MWM test showed that AI-treated animals (mice) displayed no impairment in spatial memory and learning in the reference version of the test. During the spatial reference memory (SRM) test, the AIC13 treated mice displayed latency in comparison with the mice in the control groups in reaching the stationary hidden platform marked by visible cues. However, since the SRM version of the MWM test does not require development of spatial mapping strategy for its solution (Morris *et al.*, 1988; Brandeis *et al.*, 1989; Capriole *et al.*, 1991), in the present study it was required that the animal is started a different randomly chosen quadrant, thus the animal can no longer rely on finding the hidden platform in relation to one cue. The animal had to build a cohesive spatial map of the room

in order to be able to find the platform. The results showed a significant impairment in spatial memory and learning indicated by a decrease in the escape latency of the AlCl<sub>3</sub>-treated mice compared with the controls. The findings in this study are consistent with previous reports (Miu *et al.*, 2003; Roig *et al.*, 2006) which have shown a spatial memory deficit in mice and rat after being exposed to Al. Therefore, regarding that AlCl<sub>3</sub> (1 mg/ml) treatment for a period of 8 weeks leads to destruction of the spatial memory in retention stage, it may be concluded that this stage is likely sensitive to acquisition and the treatment with AlCl<sub>3</sub>.

Different systems play role in the phenomenon of learning one of them being the cholinergic system. Many investigators confirm the role of the cholinergic nervous system in the process of learning and memory (Levy *et al.*, 1991). According to the preceding studies, intracerebro ventricular injection of Al leave a great effect on the reduction of the acetyl-choline transfrase (chat) in the brain cortex and the hippocampus. Studies on human brain have clearly demonstrated the association of acetylcholine and memory. For instance in AD patients it is observed that the level of acetylcholine in the brain is reduced (Bolkand, 1996). Considering the important function of the hippocampus in the spatial memory, damage to the cholinergic system in this region of the brain leads to memory deterioration (Halliwell and Gutteridge, 1995). Therefore, destruction of the cholinergic fibers can be one of the mechanisms for the damage of the spatial memory induced by AlCl<sub>3</sub>.

There is a specific system in human body which is developed to solely reconcile the damages caused by the free radicals called antioxidant defense system. Under normal conditions this system can provide an antioxidant balance between the production of free radicals and body's defense systems. When, by any means such as the weakness of antioxidant defense system, this balanced state is disturbed or the production of free radicals increases, a condition arises which is called oxidative stress (Halliwell and Gutteridge, 1995). Because the free radicals can directly measured due to their short half lives, the state of the oxidative stress is indirectly evaluated by gauging the damage induced by the free radicals. Since oxidative stress increases the oxidation level of the Pro in mice exposed to Al, one method for measuring the oxidation of Pro is to measure the level of the plasma thiol groups which decreases at the oxidative stress state. In fact, Al causes production of the free radicals which in turn causes oxidation of the thiol groups present in the plasma proteins. Moreover, the activity level of the antioxidants of the plasma gets reduced by the oxidative stress. The result of analysis of the blood samples from the Al-treated test groups corresponding to the time courses of 4 and 8 weeks showed a significant difference between the test and the control groups in both the FRAP and THIOL methods. This significant difference

is an indication for Al-induced oxidative stress in the animals belonging to the test groups.

To people who are chronically exposed to Al, it is recommended that in order to reduce the level of the free radicals in their bodies and strengthen antioxidant defense system of their bodies, and reduce the oxidative stress, consume more fresh fruits and vegetables. It is even recommended that they even take the allowed dosage of antioxidant supplements, so that in the long run, they are not inflicted by amnesia or forgetfulness.

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