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Aluminium chloride-induced Alzheimer's disease and inflammation in the male rat reproductive system

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Abstract

Background and Objective: The abundance of aluminium (Al) along with its widespread use by humans has caused problems such as Alzheimer's disease and inflammation. We investigated the inflammation of the reproductive system of Alzheimer's rat model induced by aluminium chloride (AlCl3: Abbreviated as Al) in drinking water.

Materials and Methods: Animals (200-250 g) were randomly divided into control and treatment groups with Al (10 and 50 mg/kg). They were studied for two to four weeks, during which they consumed tap water or water containing the desired volume concentration of the substance. Blood samples were taken from anesthetized animals and testicular tissue was studied with different staining methods. Serum levels of testosterone (T), LH, FSH, TNF-α and IL-1ß were analyzed. T receptor density was also studied by immunohistochemistry (IHC). The data were analyzed using analysis of variance (ANOVA).

Results: The destructive effect of Al on the testis tissue was very significant in comparison to the control, regardless of the dose and duration of use. T and TNF-α levels as well as T receptor density were decreased. However, the weight of the animals did not show significant changes during the study.

Conclusion: Al can cause inflammation of the reproductive system and disrupt the structure and function of the reproductive system of rats.

Keywords: Alzheimer's, Aluminium chloride, Inflammation, Reproductive system

1. Introduction

Alzheimer's disease (AD) is a mental disease that slowly destroys thinking skills and is the common cause of memory loss in the elderly population. Researchers have not yet discovered the main cause of this disease, but their findings show that in these patients, oxidative stress can lead to inflammation, which causes the disease to progress (1, 2).

Aluminium (Al) is widely found in nature and used by human being and accumulates in Alzheimer's sensitive nerve centers. There is very strong experimental evidence for the hypothesis that Al significantly contributes to Alzheimer's, and for this reason, appropriate trials should be taken to reduce human exposure to it (3). One of the areas where the most damage is caused by Al accumulation is the hippocampus. Due to the similarity of Al to iron, Al can cross the blood-brain barrier (BBB) and enter brain cells (4).

Although AD is a multifactorial disease, two mechanisms are known for its pathology so far. The first mechanism is the hyperphosphorylation of tau proteins and the second mechanism is the increase in the accumulation of amyloid plaques and that the accumulation of misfolded proteins causes oxidative stress. Although these two mechanisms are obviously involved in the development of AD, there is another pathological factor such as inflammation that should be considered (5).

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Inflammation in the male reproductive system is often caused by bacterial and viral infections and a wide range of non-infectious processes. However, the exact cause of inflammation in the male urogenital system and its potential contribution to infertility remains to be determined (6).

The aim of this study was to investigate the role of inflammation in the reproductive system of male Wistar rats as a model of Alzheimer's induced by oral aluminium chloride (AlCl3).

2. Materials and Methods

2.1. Animals

In this study, male Wistar rats (body weight: 200-250 g) purchased from Pasteur Institute of Iran were used. The animals were kept in standard cages at the Shahed University Animal Care Center at a temperature of 22±2°C and humidity (55%±15%). The dark and light cycle was 12/12 h and the rats had free access to water and food ad libitum.

2.2. Code of ethics

The local ethics committee of the university approved the ethical protocol and assigned this code to this research as IR.SHAHED.REC.1401.041.

2.3. Animal groups

Control groups one group for the two-week period (6 rats) and one group for the four-week period (6 rats): a total of 12 rats. And the group receiving aluminium chloride (Al) with a low dose (10 mg/kg): one group for the two-week period (6 rats) and one group for the four-week period (6 rats): a total of 12 rats. And the group receiving high dose Al (50 mg/kg); one group for the two-week period (6 rats) and one group for the four-week period (6 rats), a total of 12 rats.

2.4. Experimental procedure

After the treatments, the rats were anesthetized and blood samples were provided. Anesthesia was performed with ketamine (100 mg/kg) and xylazine (20 mg/kg) as an intraperitoneal (i.p.) injection and the blood sample was taken transcardially under deep anesthesia. After surgery and testicular tissue dissection, the animals were euthanized by carbon dioxide gas. The testis was fixed in 10% formalin, and after three days, testis sections (4 µm) were prepared. Tissue processing was performed with a standard processor and staining was done according to the following procedure:

Hematoxylin-eosin (H & E) staining

With the help of this staining, many tissue observations such as the destruction of testicular tissue are possible. H&E include two dyes; hematoxylin stains the cell nucleus blue-purple and eosin turns the extracellular matrix and cytoplasm pink. At first, the slides were placed in xylene for 30 minutes to deparaffinize then immersed in alcohol (96%, 80%, 70%, 50%). Then, the slices were stained by 20% hematoxylin solution (before use, this dye was filtered using filter paper to prevent the formation of sediment in the tissue). After 20 minutes the slides were washed once in distilled water and then stained with eosin for 5 minutes. After staining, the slides were placed in alcohol (50% to 96%). At the end, the specimens were immersed in xylene for twice each for three minutes. The last stage was mounting using Entellan glue and finally the slides were covered by coverslips.

Cresyl fast staining

This staining is used for specialized examination of the testicular tissue in order to show the border between the seminiferous tubules, interstitial Leydig cells and other specialized components and provides a detailed study of the seminiferous tubules. For cresyl fast dyeing, fresh dye was first prepared (0.1 g of cresyl was dissolved in 100 ml of distilled water and stirred with a magnetic stirrer for half an hour, and then the solution was filtered with filter paper). In order to deparaffinize the slides, they were placed in xylene for half an hour and then put in 96%, 80%, 70%, and 50% alcohol, each for 5 minutes. Then, the prepared cresyl dye was added on the sections. It took about 50 minutes to stain (checked often) and then washed with distilled water. Then the dehydration steps (in 50%, 70%, 80%, 96% alcohol, each for 1 minute) were performed. And finally clearing by xylene wad done twice. At the end, Entellan glue was added.

Testosterone receptor density studies with immunohistochemical staining

First, in order to deparaffinize the sections, they were placed in xylene for 20 minutes and then flooded in alcohol (96%, 80%, 70%, and 50% alcohol, each for 5 minutes). Then, the slides were placed in the phosphate buffer solution (pH=7.4) for one to two minutes, and then briefly placed (1 to 2 seconds) in the Triton-X100 solution. Later, they were exposed to specific antibody (Z2640RP) for one hour. The sample was then washed four times in phosphate buffer (1-2 minutes each time) and exposed to avidin (for 10 minutes at the lab). It was then exposed to chromogen for 30-45 minutes, then immersed once in the solution phosphate buffer and then rinsed in distilled water. After that, dehydration steps were performed (in alcohol 50%, 70%, 80%, 96%, each for 1-2 minutes). Finally, the slices were cleared by xylene twice for three minutes, and at the end, Entellan was added.

2.5. Statistical analysis

All analyzes were done with SPSS software (version 22). Kolmogorov-Smirnov test was used to confirm the normality of data and one-way analysis of variance (ANOVA) was used to measure the data. Tukey's *post hoc* test was chosen to compare the differences between groups. $P<0.05$ was considered as a significant value.

3. Results

3.1. Serum concentrations of testosterone, LH, FSH, TNF-α, and IL1β

Different groups showed changes in the serum level of these factors. The results are shown in Figures 1 to 5, respectively.

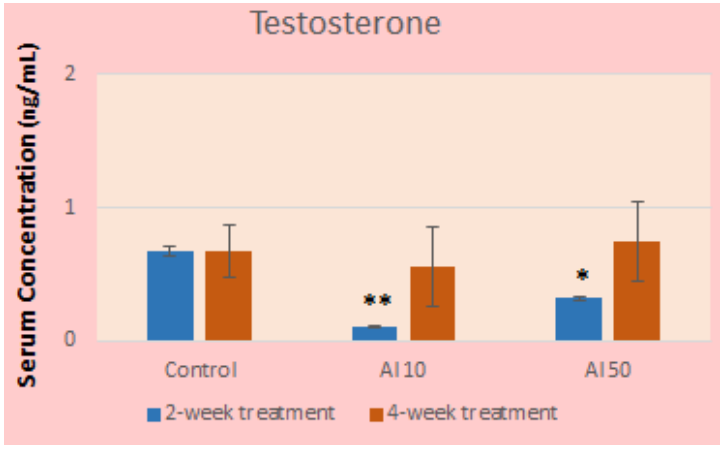

Fig. 1: This figure shows the testosterone serum concentration based on ng/ml. The control group received only drinking water. Aluminium chloride (AlCl3: abv as Al) was prescribed under 10 and 50 mg/kg (as volume concentration in drinking water) during a period from two to four weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05) (**P<0.01).

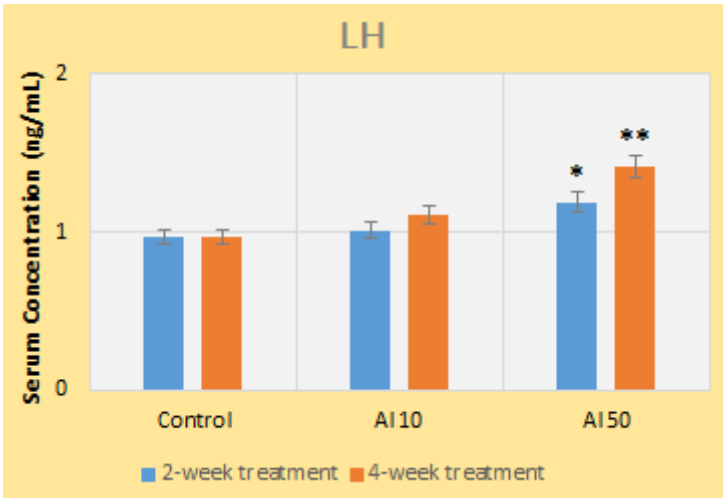

Fig 2: It shows the serum concentration of LH based on ng/ml. The control group received only drinking water. Aluminium chloride (Al) was given under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05) (**P<0.01).

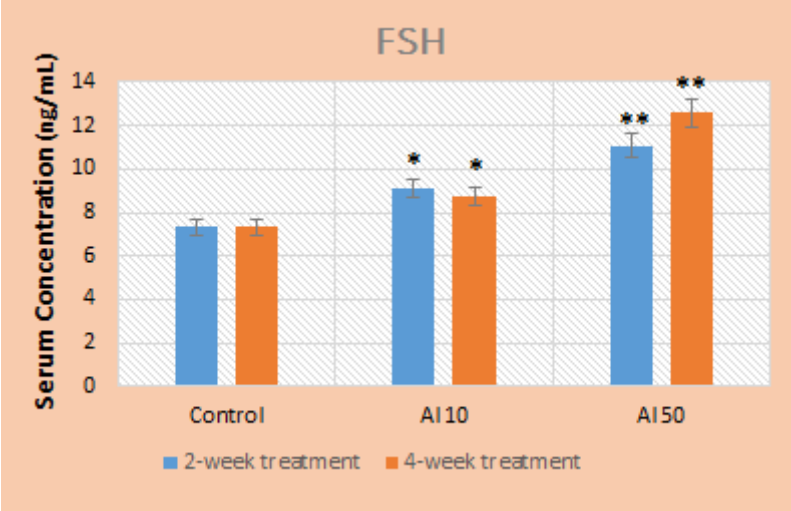

Fig 3: This figure shows FSH serum concentration based on ng/ml. The control group received only drinking water. Al was given under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05) (**P<0.01).

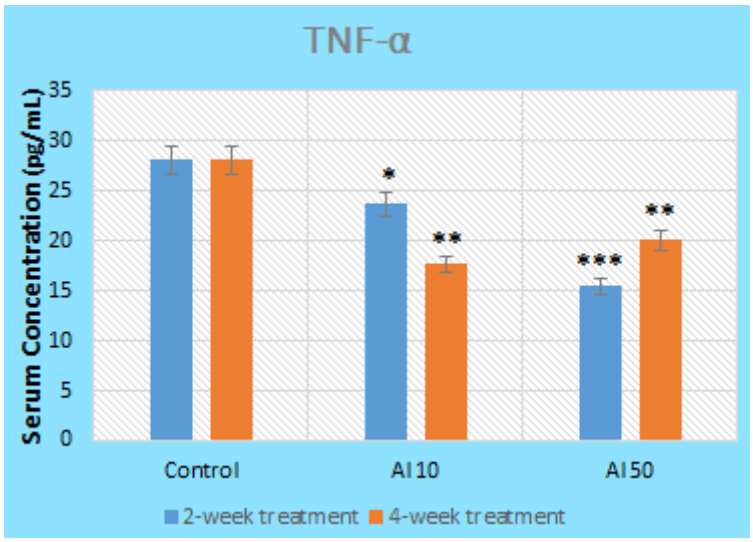

Fig 4: It shows TNF-α serum concentration based on pg/ml. The control group received only drinking water. Al was prescribed under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05) (**P<0.01) (***P<0.001).

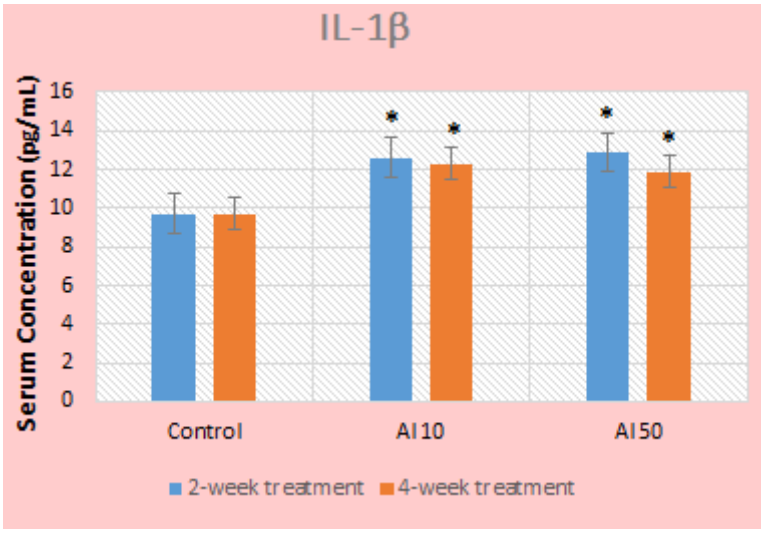

Fig 5: IL-1β serum concentration based on pg/ml is shown. The control group only received drinking water. Al was used under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05).

3.2. The results of pathological studies

3.2.1. The results of evaluation of destruction in seminiferous tubules

These findings were obtained using microscopic sections of the testes under ×40 magnification with the software ImageJ (Fig 6).

Also, Figure 7 shows the tissue results with H & E staining.

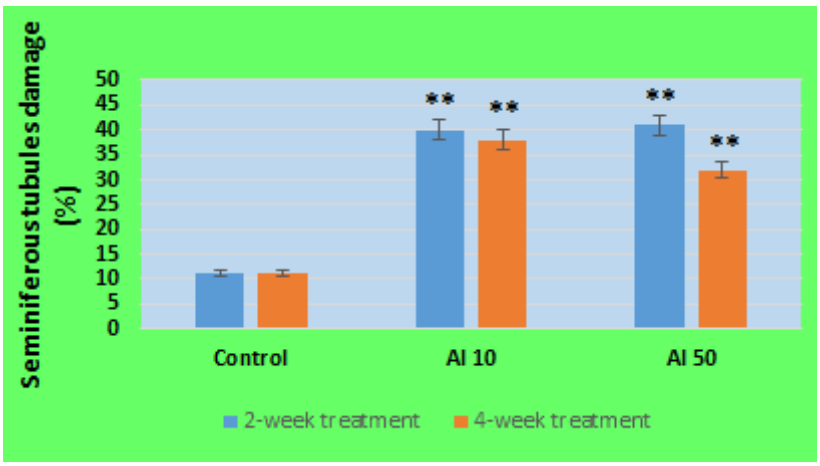

Fig 6: Destruction of seminiferous tubules. The control group received only drinking water. Al was prescribed under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (**P<0.01).

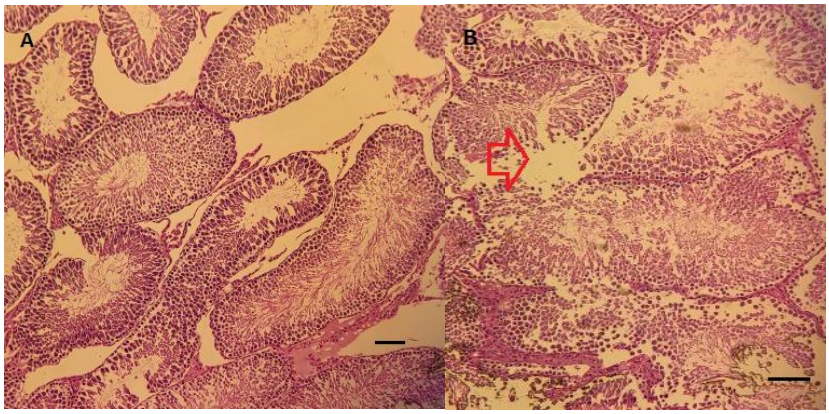

Fig 7: Transverse section of rat's testicular tissue under hematoxylin-eosin staining. The control group (A) received only drinking water. Al was used under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. The control group showed no tissue damage. In the 4-week treatment group (B), a lot of tissue damage was observed regardless of the dose. Scale line is 50 µm.

3.2.2. Lumen volume of seminiferous tubule

Using testicular microscopic sections and using ImageJ software, the samples were examined with x40 magnification at 10 random points from each tissue sample and the result was obtained quantitatively (based on percentage) and then analyzed with the help of ANOVA (Fig 8). Also, Figure 9 shows the results of Cresyl fast coloring.

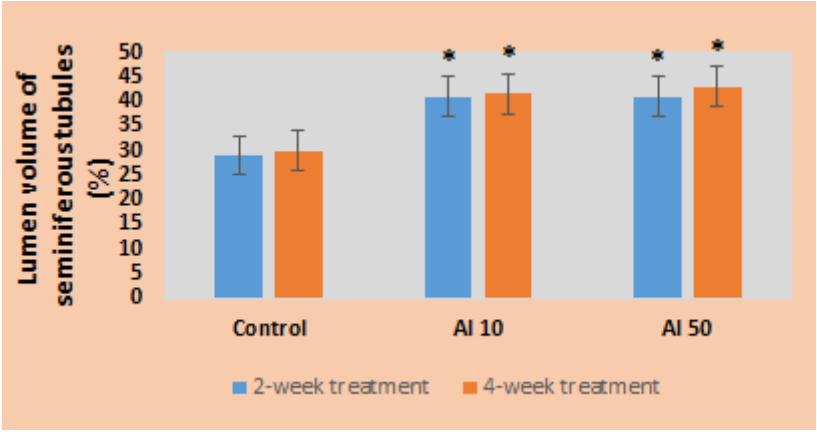

Fig 8: Lumen volume (percentage) of seminiferous tubules. The control group received only drinking water. Al was used under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Asterisks were obtained based on Tukey's *post hoc* test (*P<0.05).

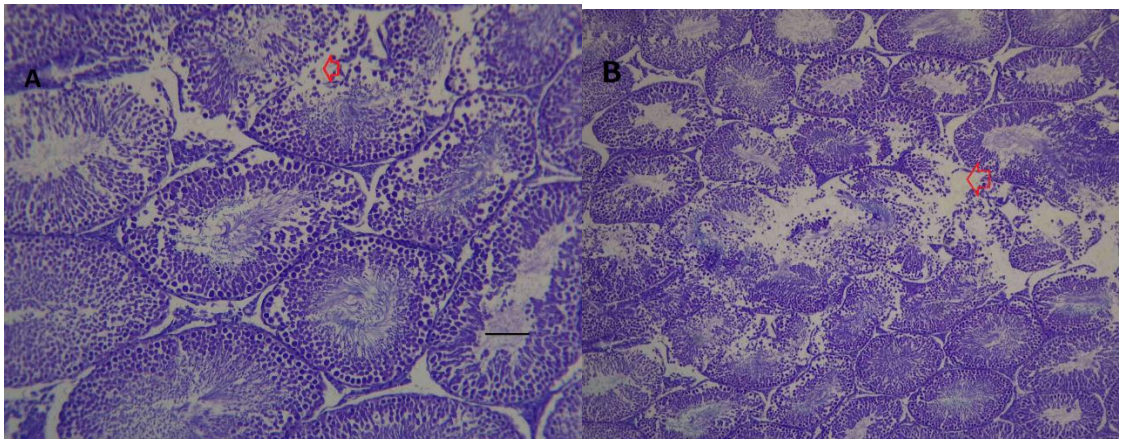

Fig 9: Transverse section of rat's testis tissue under cresyl fast staining. The control group received only drinking water. Al was given under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. Control group showed no tissue damage (A), but the damage was shown in the 4-week treatment group (B).

3.2.3. The results of testosterone receptor density studies with immunohistochemical staining

The specific staining of testosterone receptors was performed and the density of receptors in the tissue was checked. The receptor density in the control samples was standard, but a significant decrease was observed in the treatment groups, and the percentage of positive reaction was obtained with ImageJ software (Fig 10).

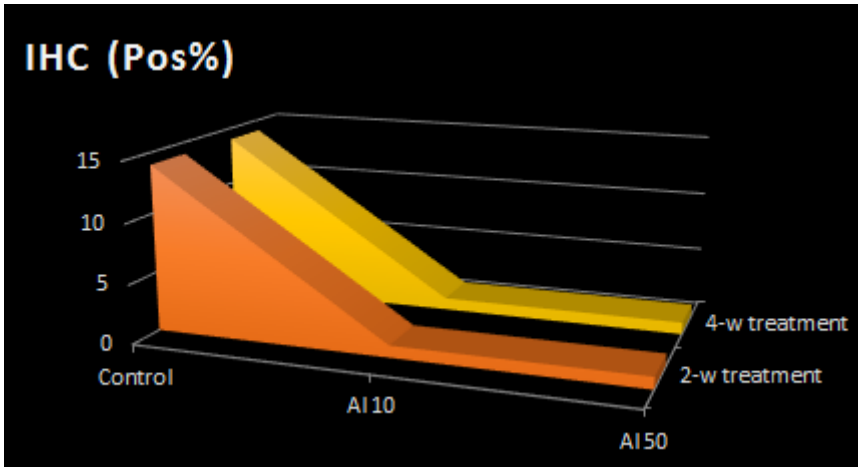

Fig 10: Percentage of positive response to immunohistochemical staining of testosterone receptors. The control group received only drinking water. Al was prescribed under 10 and 50 mg/kg (as volume concentration in drinking water) during 2-4 weeks. There was a significant difference in all treatment groups compared to the control. Also, there was a significant decrease in the density of receptors compared to the control.

4. Discussion

Alzheimer's disease (AD) is a neurodegenerative disorder that its incidence rate is increasing in the elderly. Exposure to environmental pollutants such as aluminium can stimulate or accelerate the involved mechanisms such as tau phosphorylation. The disease is the most common neurological disorder characterized by memory impairment, especially in the elderly, and is associated with neurological and neurobehavioral changes in patients. An important feature in AD is the excessive production and accumulation of amyloid beta (Aβ) peptide. This leads to large-scale neuronal death, neuronal atrophy, and loss of synapses in the final stage of the disease. Another well-known feature of AD is the reduction of cholinergic activity, especially in the limbic system, including the hippocampus. It is well established that a decrease in cholinergic markers and an increase in enzyme-related acetylcholine degradation occur in AD. Although genetics play a key role in the occurrence of AD, some environmental factors such as toxins and air pollutants can stimulate or accelerate its development. A group of environmental pollutants consists of heavy metals. Using aluminium metal is one of the ways to create Alzheimer's model in laboratory animals (18). In the present study, aluminium chloride (AlCl3: abv as Al) was given orally in drinking water to animals. This method is less expensive than Aβ injection and less invasive than gavage. According to the results of this study, the testicular tissue of Al-treated rats showed significant tissue damage compared to the control. Also, the density of Leydig cells decreased and the luminal space of the tubules increased. Due to the use of different doses of Al, testosterone (T) levels decreased, but gonadotropin factors and IL-1ß levels increased. According to the previous research conducted in the Alzheimer's experimental model, a decrease in the level of T hormone has also been reported. It has also been shown that 12 weeks of aerobic exercise causes a significant decrease in corticosterone (P=0.006) in Alzheimer's rats. It seems that the aerobic exercise, by increasing T levels and decreasing corticosterone, is effective on depression-related behaviors in rats with AD (19). Also, according to another research, additional form of stress (exposed to sound for 50 days with an intensity of 90-120 dB and a frequency of 300-350 Hz) was imposed on the animal and the T level decreased (9). Aerobic exercise can cause a lot of physiological stress and by increasing the level of cortisol, a hormone that inhibits the production of T, the T decrease occurs (20). In a study aimed at investigating the effect of Fipronil-Induced Oxidative Stress (FIP) on fertility in rat, it was observed that this process causes a decrease in T levels (21). With serological studies in male rats, Saki and his colleagues stated that the decrease in T increases gonadotropin factors (9). In another research conducted by Ghayumi and his colleagues in 2015, to investigate the effect of weightlessness on the function of male rat gonads and to determine the secretion of T and LH hormones (by simulating microgravity conditions using special cages), a decrease in T and an increase in LH secretion were observed (12).

Although the above mentioned studies were not done in this study, it can be stated that due to the disorder in the testicles and the impossibility of proper feedback from the steroid factors secreted by this gland, the pituitary gland can change the secretion of gonadotropins (increase in FSH). However, two inflammatory factors levels were checked: tumor necrosis factor alpha (TNF-α) decreased but interleukin 1β (IL-1β) increased. In a previous study aimed at investigating the effect of fibroblast growth factor (FGF) peptide antagonist on murine breast tumor growth, and by measuring serum levels of interleukin 8 and TNF-α, similar findings were reported on cytokine levels (13). In a study, 70 female Sprague rats with an average weight of 250 ± 10 g were used with the aim of investigating the effect of gallic acid as a possible protective factor on the TNF-α level in the hippocampus, and according to their finding, the level of TNF-α in the hippocampus of the treated rats showed a significant decrease compared to the Alzheimer's group (14). Of course, in that study, the animal strain was different, and the estrous cycle of the animal was not determined.

In the present study, the level of IL-1β increased. It has been shown in other studies that this factor is effective as a main pro-inflammatory and inflammatory factor in the occurrence of inflammation, which confirms the present results. According to a previous study, IL-1β, IL-6 and TNF-α are the cytokines that play a role in inflammation. IL-1β is an immunoregulatory cytokine that is overexpressed in the cortical region of the damaged brain of AD. Both IL-1β and Aβ precursor protein have been reported to be significantly increased in individuals with cognitive impairment due to epilepsy, which may predispose these individuals to early AD pathological changes (15).). Although the expression of most cytokines is normal in healthy tissues, the IL-1β level increases in some neurodegenerative diseases. Also, some researchers have found that regular sports activities, especially aerobics, are effective in reducing its level (16,17).

In the histological study of the testis regarding the volume of the lumen of the seminiferous tubules, destruction of the seminiferous tubules and increase in the lumen volume was obtained. In a study conducted by other researchers with the aim of investigating the cytotoxic effects of ciprofloxacin in the testis tissue of rat, similar results have been reported (18). In other studies, extensive degeneration of germinal tissue and atrophy of Leydig cells have also been indicated due to severe reduction of T (19,20). Therefore, the decrease in the germinal layer and the increase in

ductal space that we observed in present study may be related to the reduction of T.
In relation to the results of testicular tissue staining obtained in this study, it is better to refer to tissue studies with different concentrations of pheromone, which had a relatively significant effect on the numbers of spermatocytes and Leydig cells (20). In line with the above studies, Al has a noticeable effect on the cell density of both the germinal layer and the other cell types. Based on cresyl fast staining with the aim studying the interstitial space, the differences between the control and Al-treated samples were statistically significant. It was mentioned that this staining is specific to the examination and observation of tissue necrosis and of course that apoptotic events were not noticeable in this study. Immunohistochemical staining was one of the most specific measurements by which we achieved a statistically significant difference between the control and Al-treated samples. In a research conducted in 2013 (21), on the role of androgen receptors in rat, similar finding was also pointed out. It should be noted that T produces its inductive physiological effects after binding to the receptor. Therefore, many of the results seen in this research can be attributed to the decrease in the level of these receptors.
In the present study, there was no significant change in body weight and no change in the dimensions of the testes were observed (data not shown).

Conclusion

It seems that the long-term use of aluminium chloride along with Alzheimer's disease can cause inflammation of the genitourinary system due to its destructive effect on the testicular tissue and the reduction of testosterone levels, as well as the stimulation of the secretion of pro-inflammatory factors.

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