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Full Length Research Paper

Aluminum-induced testosterone decrease results in physiological and behavioral changes in male mice

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Recently, there has been much controversy on the role of testosterone on social and aggression behaviors. This work aimed to determine the effect of testosterone decrease, induced by aluminum exposure on the level of aggression. Male Swiss-Webster strain mice were classified into three groups. The first (control group) received distilled water, while the second and third groups were administrated 300 and 600 mg/kg aluminum chloride, respectively, by oral route for 20 days. Thereafter, they were subjected to "standard opponent" test. A significant decrease in testosterone levels in the treated groups was obtained at both the low and high doses of aluminum. Expectedly, significant decreases were observed in the social contacts, threat, attack and number of fights of both treated groups in a dose dependant manner. All blood parameters revealed a dose dependent significant decrease as well. A significant decrease in both serotonin and dopamine levels was simultaneously obtained with the decrease of testosterone level especially at the high dose of aluminum. In contrast, at the high dose, acetylcholine recorded significantly high value. In conclusion, aluminum-induced testosterone decrease resulted in a significant decline in aggression, several blood parameters and levels of neurotransmitters.

Key words: Aluminum, Swiss-Webster mice, standard opponent test, social behavior, testosterone.

INTRODUCTION

Testosterone is primarily produced in the testes and the ovaries, although small amounts are also produced by the adrenal glands. It is the main male sex hormone and an anabolic steroid. The brain is an important site of action for testosterone (Rommerts, 2004). It is demonstrated that testicular secretions are necessary for aggressive behavior in roosters. Increased aggression was observed in males exposed to anabolic androgenic steroids, which are independently of treatment age (Salas-Ramirez et al., 2009; Çetin and Çilden, 1996). Research on the neuro-endocrinology of aggression has been dominated by the paradigm that the brain receives gonadal hormones, primarily testosterone, which modulate relevant neural circuits (Soma et al., 2008). Testosterone plays a role in aggressive behavior, but the mechanisms remain unclear.

Testosterone increases the propensity towards aggression because of reduced activation of the neural circuitry of impulse control and self-regulation (Mehta and Beer, 2009). Testosterone is converted into 5-alpha-dihydrotestosterone which acts on androgen receptors, or when converted to estradiol by the enzyme aromatase, acts on estrogen receptors. There is overwhelming evidence that most of the effects of testosterone in mediating aggression occur after aromatization (Schlinger and Callard, 1990).

Despite the fact that many studies proved the correlation between testosterone and aggression, this relationship needs to be clarified. Impairment of testosterone can be caused by aluminum accumulation in the testis, sperms and seminal fluids (Dawson et al., 1998; Guo et al., 2001). A decrease of testosterone was previously obtained using aluminum in several studies on animal models (Llobet et al., 1995; Bataineh et al., 1998; Guo et al., 2001,

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Abbreviations: HPLC, High performance pressure liquid chromatography; **EDTA,** ethlenediaminetetraacetic acid.

2005). In the present study, a decrease of the testosterone level using aluminum chloride was induced and then, the different behavioral and physiological parameters were tested to investigate such relationship.

MATERIALS AND METHODS

Experimental animals

Adult Swiss mice weighing 25 to 30 g were obtained from the animal house in the Faculty of Pharmacy, King Saud University, Saudi Arabia and housed in stainless steel wire cages (1 animal/cage) under pathogen-free conditions. Animals were maintained at 18 to 22°C on a 12:12 h light/dark cycle and provided with food and water *ad libitum*. Male Swiss-Webster strain mice were classified into 3 groups. The first (control group) received distilled water, while the second and third groups were administered 300 and 600 mg/kg aluminum chloride, respectively, by the oral route for 20 days. Thereafter, they were switched to plain tap water and behavioral tests were conducted.

Estimation of testosterone

Blood was obtained by a thin capillary tube from the Retro-orbital plexuses of the test animal. Blood without any anticoagulant was centrifuged at 4000 rpm/min for 10 min and plasma was obtained and kept at -30°C until it was used for hormone estimation. Testosterone was estimated using hormone analysis instrument (Hitachi-Eleceys 2010-Roche Diagnostic, USA) by the method of electrochemiluminescence immunoassay.

"Standard opponent" tests

Male subjects from each litter, and in all 14 male subjects from each group, were separately housed for 14 days. After this isolation period, the males from each group were made subjects to the standard opponent test under dim red lighting (ca. 8 lux) as previously described by Brain et al. (1989). The docile and age-matched males "standard opponent" were rendered anosomic by applying 25% zinc sulphate tract under ether anaesthesia for 1 h prior to encounters. The anosomic "standard opponent" intruders were put into the home cages of the test animals and the standard opponent test of each animal was observed for 500 s. The opponents were used only once and the selected elements of behavior namely, naso-genital and naso-nasal contacts, threat, attack and the numbers of fights were studied (Ajarem and Brain, 1993).

Blood parameters

Blood was collected from animals within heparinized tubes at the end of the experiments. Blood parameters namely, red blood count, packed cell volume, hemoglobin content, total white blood count, differential counts and blood platelets were measured using the automated parameter hematology analyzer (T 450, USA).

Estimation of dopamine and serotonin

Dopamine and serotonin were estimated in the brain homogenate according to the method of Patrick et al. (1991) using high performance pressure liquid chromatography (HPLC). Brain tissue was homogenized in 0.1M perchloric acid containing 0.05% ethlenediaminetetraacetic acid (EDTA) and isoproterenol (1 µg/ml 0.1N PCA). Homogenate was then centrifuged for 5 min at 17000/min and the supernatant was filtered (0.45 µm) to be subjected onto HPLC.

Estimation acetylcholinestrase

Brain of the tested animals was removed and gently rinsed in physiological saline (0.9% NaCl) and then blotted in whatman filter paper. The organ fresh weights were recorded and frozen until use. A 10% (w/v) homogenate of each frozen tissue was prepared and the supernatant was applied for enzyme assay. The acetylcholinestrase activity in the homogenized brain tissue was estimated by using acetyl chloride as the substrate. The specific activity of acetylcholinestrase was expressed as micromoles acetylcholine chloride hydrolysed per gram wet tissue weight per hour at 37 ± 1°C.

Statistical analysis

The data were normally distributed and therefore, they were compared within the experimental groups by the analysis of variance (ANOVA) using minitab computer programme. Data were subsequently analyzed by Student's t-test (Yamane, 1973) for biochemical analyses and Mann-Whitney U tests (Sokal and Rohlfe, 1981) for behavioral analyses.

RESULTS AND DISCUSSION

This work was planned to investigate the behavioral and physiological changes induced in male albino mice associated with the decrease of testosterone level which resulted from the exposure to aluminum chloride. Results showed that the testosterone hormone was dramatically decreased with the increase of the aluminum chloride dose. Testosterone recorded a significant decrease at the low dose of aluminum chloride (Figure 1). As aluminum chloride dose increased, testosterone recorded a further significant decrease ($p < 0.01$).

Based on the fact that blood parameters are closely related to the physiological changes, they were studied as well. Red blood count, packed cell volume and hemoglobin content showed a decline in their values at the low dose of aluminum and low level of testosterone. The decline in all their values was significantly increased at the high dose of aluminum as the testosterone level became significantly low (Figure 2). On the other hand, it was shown that the total white blood cell count and the white differential count (Figure 3A and B) recorded a clear change when compared to the values obtained for the control mice. A significant decrease in the total blood cell count was observed in a dose dependent manner. Also, in the differential count, neutrophil recorded some significant low values when compared to the control. On the contrary, a significant increase in the lymphocyte count was recorded as shown in Figure 1B.

The neurotransmitters levels are good indicators for the behavioral changes. Figure 4 shows that the levels of dopamine and acetylcholine were significantly decreased

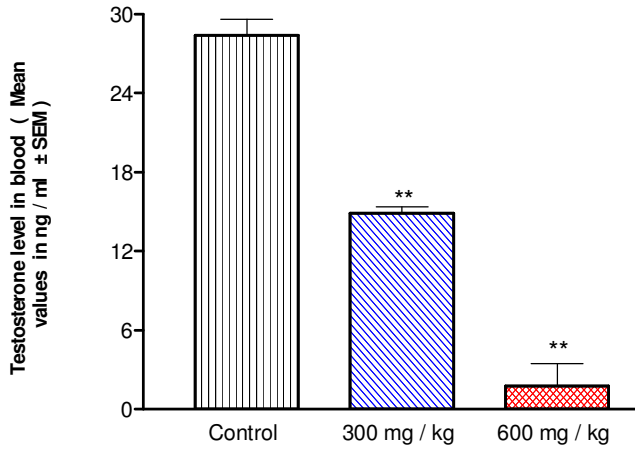

Figure 1. Testosterone levels induced by using two doses of aluminum chloride, 300 and 600 mg/kg, respectively, by the oral route for 20 days. Student Newman-Keuls multiple comparison test by ANOVA since the (**) shows statistically significant ($p < 0.05$).

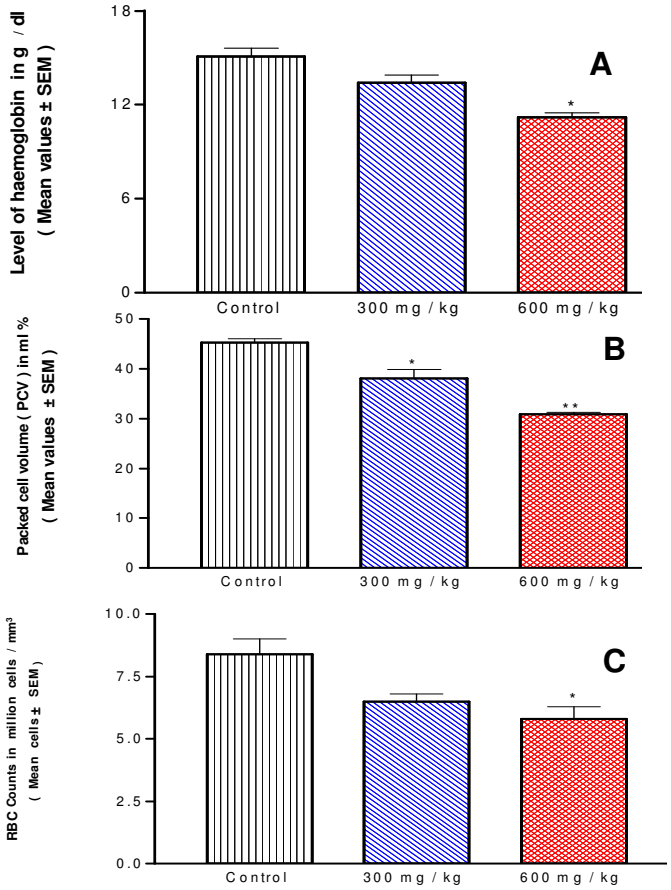

Figure 2. Some blood parameters (A: level of hemoglobin content, B: packed cell volume and C: red blood cell count) induced by the aluminum administration and its subsequent testosterone low levels. Student Newman–Keuls multiple comparisons test by ANOVA. (* and **) show statistically significant ($p < 0.05$ and $p < 0.01$, respectively).

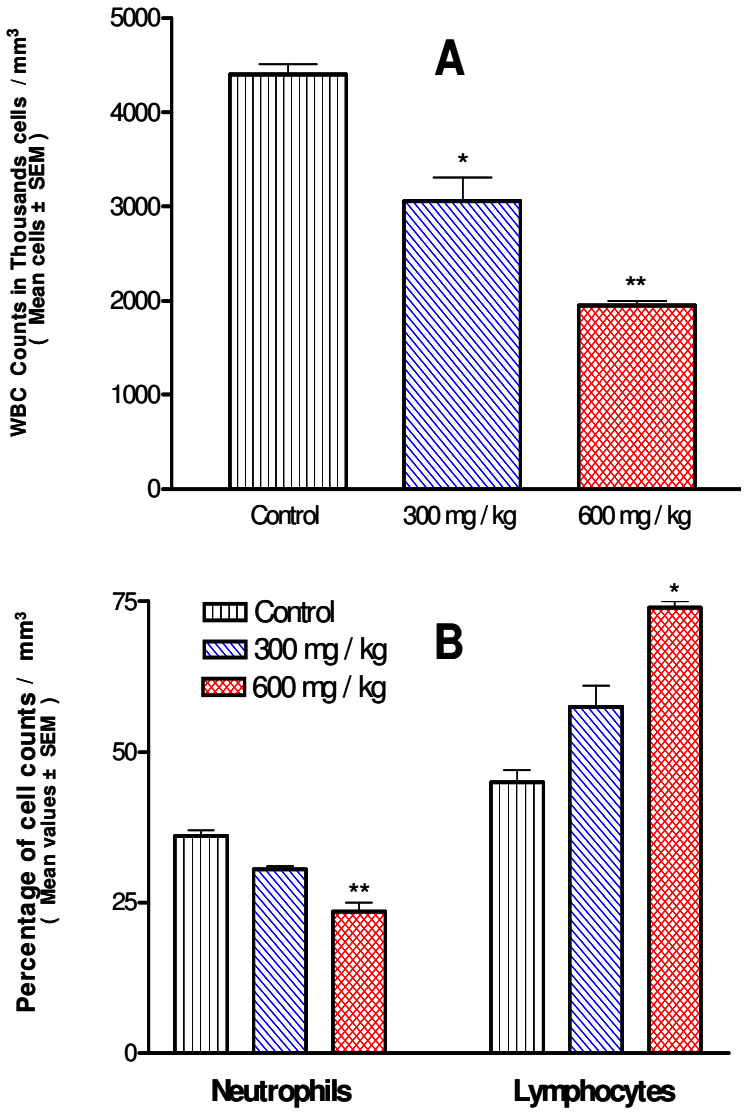

Figure 3. Some blood parameters (A: total white blood count, B: differential count) induced by the aluminum administration and its subsequent testosterone low levels. Student Newman–Keuls multiple comparison tests by ANOVA. (* and **) show statistically significant ($p < 0.05$ and $p < 0.01$, respectively).

($p < 0.05$) at the low dose of aluminum chloride and with the decrease of testosterone. At the high dose of aluminum and the further low level of testosterone, dopamine recorded further a highly significant depletion ($p < 0.01$) when compared to both the control and the second groups. On the contrary, acetylcholine recorded significant increase at this high dose of aluminum. Results showed that, at the low level of testosterone, serotonin recorded a clear but non-significant decrease, while it was significantly decreased with further decrease of the testosterone.

From the data presented in Tables 1 (A and B), it is shown that the latency to threat and fights was significantly increased in a dose dependent manner when compared to the control mice. The threat in the treated groups decreased from 23.15 at the control to 15.85 and 12.00 at the low and high doses, respectively. Accordingly, the latency of threat was increased from 10 to 90 and 425 s. Similar results were obtained for the attack activities. The number of fights was highly significantly decreased to 6.5 from 18.5 of the control at the high dose of aluminum. Social and non-social activities were significantly decreased and increased, respectively, in a dose dependent manner when compared to the control mice. From these results, there was a clear correlation between the testosterone low levels and all social, non-social and aggression activities.

The decreased levels of testosterone are associated

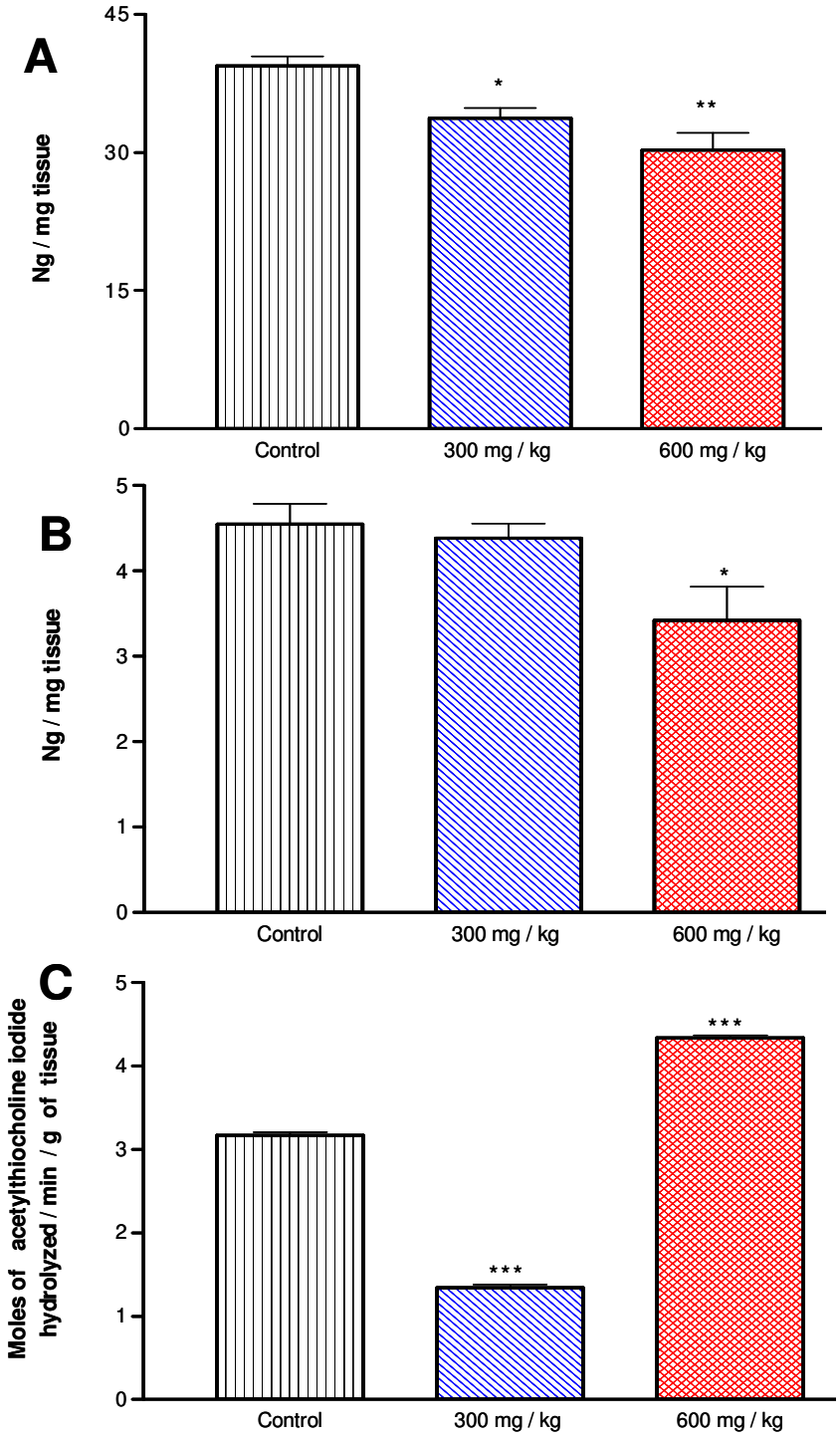

Figure 4. The neurotransmitters changes induced by two doses of aluminum chloride, showing the level of dopamine (A), serotonin (B) and acetylcholine (C). Student Newman–Keuls multiple comparisons test by ANOVA. (*, ** and ***) show statistically significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

with an increase risk for the development of many diseases such as type 2 diabetes, cardiovascular diseases, obesity, depression, anxiety and Alzheimer's disease, and the increased risk of early mortality (Hogervorst et al., 2005; Nieschlag, 2006; Rosario and Pike, 2008). Also, it was previously proved in the study's work that the decrease of testosterone impaired learning and memory of animal models (Taweel, 2009).

Table 1. A and B, The social behavior of male albino mice induced by exposure to the aluminum chloride and its subsequent decrease of the testosterone hormone.

A

Group	Testosterone level (ng/ml)	Median number (with ranges) of seconds allocated to behaviours					
		Nonsocial investigation	Social investigation	Defense	Threat	Attack	Displacement
Control	28.400 ± 1.200	125.25 (84.30 – 171.20)	228.50 (214.10 – 242.40)	17.95 (8.20 – 20.70)	23.15 (13.30 – 35.30)	62.40 (45.80 – 100.20)	26.50 (4.60 – 56.60)
300 mg/kg	14.900** ± 0.500	244.90*** (212.00 – 310.40)	160.80 *** (95.40 – 218.50)	24.50 (12.30 – 52.30)	15.85 * (10.00 – 23.70)	22.00 *** (14.30 – 45.30)	24.70 (16.20 – 60.90)
600 mg/kg	1.800 ** ± 1.700	311.15 *** (250.50 – 400.00)	100.50 *** (30.50 - 159.80)	26.80 * (16.00 – 40.30)	12.40 ** (8.50 – 26.40)	12.05 *** (0.00 – 30.40)	32.85 (20.40 – 41.00)

B

Group	Testosterone level (ng/ml)	Median number (with ranges) of acts and postures						
		Latency to threat (sec)	Latency to attack (sec)	Number of fights	Number of naso-nasal contacts	Wall rears	Rears	Latency to threat(s)
Control	28.400 ± 1.200	10.00 (3.00 – 30.00)	25.00 (10.00 – 70 .00)	18.50 (13.00 – 21.00)	25.00 (20.00 – 29.00)	17.50 (14.00 - 22.00)	12.00 (13.00 - 29.00)	10.50 (8.00 - 13.00)
300 mg/kg	14.900** ± 0.500	90.00** (30.00 – 200.00)	155.00 ** (100.00 - 350.0)	11.50 ** (9.00 – 18.00)	16.50*** (10.00 – 19.00)	11.50* (8.00 - 19.00)	20.00 ** (13.00 – 29.00)	14.00* (10.00 – 28.00)
600 mg/kg	1.800** ± 1.700	425.00** (200.00 - 490.00)	358.00 *** (0.00 – 490.00)	6.50 *** (0.00 – 13.00)	6.50 *** (4.00 – 11.00)	32.00 *** (25.00 – 53.00)	32.00 *** (25.00 – 53.00)	23.50*** (14.00 - 36.00)

* , ** and *** shows statistically significant differences at P < 0.05 , P < 0.01 and P < 0.001, respectively from the control by Mann-Whitney U test.

Data of the present work cleared an obvious delay of the aggression at the low levels of testosterone. Thus, aggression was shown to be related with testosterone level without clear effects of any alternative pathway. This was proved by increasing the latency of aggression with the further decrease of testosterone level. Similarly, many studies showed a close relationship between testosterone and different behavioral changes, especially aggression (Schlinger and Callard 1990; Zitzmann, 2006; lynn, 2008). In contrast, Soma et al. (2008) mentioned that some species show aggression with low levels of testosterone and they suggested an alternative pathway for this aggression.

Animal studies provide a strong evidence of change, serotonergic neurotransmission, being associated with change aggression (Manuck et al., 2002; De Almeida et al., 2005; Ferrari et al., 2005). Both serotonin and testosterone have been implicated in the regulation of aggression. However, findings in humans are inconclusive, with respect to the main effects of either system. Animal models implicate testosterone to modulate serotonin system activity and the behaviorally relevant interactions of testosterone and serotonin with respect to aggression (Kueppera et al., 2010). The testosterone level would explain the variance in central serotonin activity since it modulates the expression of several subcomponents of the serotonin synapse (Zhang et al., 1999; Fink et al., 2009), and this appear to be dependent on its conversion to estrogen by aromatase (Schlinger and Callard, 1990; Fink et al., 1999). The study's data obviously revealed that the serotonergic system was modulated with the change in testosterone level.

Elaine et al. (1997) reported that steroid hormones prime neural circuits for sexual behavior, in part regulate enzymes, receptors or other proteins affecting neurotransmitter functions. A positive correlation was obtained between dopamine

content and plasma testosterone levels in marmosets (De Souza Silva et al., 2007, 2009). Testosterone is up-regulating nitric oxide synthesis, which in turn enhances dopamine release (Elaine et al., 1997). The two major metabolites of testosterone, 17h-estradiol and dihydrotestosterone, affected the extra-cellular dopamine (Putnam et al., 2003). The former stimulates nNOS and increases NO production, which facilitates dopamine release. The latter enhances sensory inputs to certain region in the brain, which contributes to acute stimulation of nNOS. It is quite clear that such steroidal modulation influences the development of hypothalamic dopamine cells (Simerly et al., 1997) and this subsequently, explained the change of dopamine obtained in the present study and its subsequent low aggression.

In behaving animals, extracellular acetylcholine levels in the hippocampus are known to increase during stress (Imperato et al., 1991; Tajima et al., 1996; Mizuno and Kimura, 1997); and this can explain the significant increase of acetylcholine with the further increase of the dose in the present work.

Regarding the change in the studied blood parameters, a study of Riedmaier et al. (2009) showed that testosterone, in part, influences mRNA expression of 6 selected genes out of 37 in the whole blood. High testosterone levels increased erythrocyte number and blood hemoglobin content. It also altered leukocyte number and increased plasma proteins in adult birds (Puerta et al., 1995). Thus, it is more likely that the decrease of testosterone might cause the obtained decrease of approximately all the studied blood parameters. Concerning the increase in lymphocyte number, Genhong et al. (2003) mentioned that androgens influence some immunological processes, including alternation of the number and function of the circulating lymphocytes and monocytes. It is logical that the decrease of blood parameters can directly affect the animal activities and in particular, the animal aggression behavior.

In conclusion, the decrease of testosterone hormone in male albino mice induced a clear delay and an obvious decrease in the aggression behavior with some other social activities. Testosterone decrease might negatively affect the aggression behavior via decreasing the related neurotransmitters, especially dopamine and serotonin. The testosterone suppression of different blood parameters might also play an effective role in this latency and low aggression. This study confirmed the correlation between the testosterone level and aggression by impairment of many physiological intermediates. However, it is possible also that aluminum resulted in an overall direct decrease in the behavioral changes as found by Golub and Germann (1997) and Bataineh et al. (1998). Further future studies should investigate these issues to declare the mechanism of the aggression behavior. In general, this study may be an approach to mimic the physiological changes seen in aged people, since it revealed an intricate interplay between behavior and hormonal changes.

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