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Aluminum Chloride Causes the Dysfunction of Testes Through Inhibiting the ATPase Enzyme Activities and Gonadotropin Receptor Expression in Rats

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Abstract The aim of this experiment is to explore the effects of aluminum chloride ($AlCl_3$) on the ATPase enzymes and gonadotropin receptors in the testes. Eighty male Wistar rats were orally exposed to 0 mg/kg body weight (BW) (control group, CG), 64 mg/kg BW (low-dose group, LG), 128 mg/kg BW (mid-dose group, MG), or 256 mg/kg BW (high-dose group, HG) for 120 days. The microstructure and ultrastructure of testes; the activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase; and the mRNA and protein expressions of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptors (LHR) in the testes were examined. The results showed that the testes histological structure were damaged; the activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase, the mRNA and protein expressions of FSHR and LHR in the testes were all decreased in the rats with $AlCl_3$ exposure. It indicates that $AlCl_3$ causes the dysfunction of testes in rats.

Keywords Aluminum chloride·rat · Testes · ATPase enzymes · Gonadotropin receptor

Introduction

Human male infertility is a major health problem that affects approximately 10 to 15% of couples worldwide [1]. Male infertility, as the most difficult form of infertility, is governed by a variety of causative factors, including environmental disruptors, genetic defects, physiological and endocrine failure, and testis pathologies [2]. Despite 60–75% of male infertility are idiopathic, the disease is usually accompanied by qualitative (asthenospermia, teratozoospermia, and necrospermia) and quantitative (azoospermia, cryptozoospermia, and oligoasthenozoospermia) abnormalities [3, 4]. Aluminum (Al) exposure is one of the important pathogenesis for male infertility [5]. Al, as the third most abundant element on Earth's crust, is widely used in Al containers and utensils, medicines, water purifiers, and food additives, which induces a global public health problem [6, 7]. Aluminum trichloride ($AlCl_3$) (34 mg/kg body weight (BW); 25 mg/kg BW) impaired the structure of testes and epididimis in the rat and mice, respectively [8, 9]. Results obtained from Yousef et al. [10] revealed that rabbits which were orally administered $AlCl_3$ at 34 mg/kg BW every other day for 16 weeks exerted significant decrease in sperm activities and numbers [10]. Ige and Akhigbe [6] suggested that $AlCl_3$ exposure caused male rats infertility via oxidative damage. These evidences demonstrate that Al is a potential risk for male infertility, but the investigation on the ionic transport or gonadotropin receptors in male rat testes exposure to $AlCl_3$ are lacking.

One of the major mechanisms behind toxic effects of Al on reproductive system has been attributed to ionic disorders.

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ATPases, which constitute a major category of ion transporters in the human body, have a variety of significant biological and pathological roles. ATPases can maintain proper function of the vasculature through regulating the transmembrane ionic balance [10], and many studies have shown that ionic disorders in the vasculature give rise to testes dysfunction. With sublethal dose (3.5 mg/kg BW) of Al acetate, total ATPase activity decreased in testes of albino mice [11]. Our previous research observed that chronic $AlCl_3$ administration decreased ovary Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase activity; intracellular energy production; and incomplete cell membrane [12]. However, whether there is a similar effect in rat testes needs to be explored.

The follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) were expressed on sertoli cells and leydig cells, and their activation promoted the support and nourishment of germ cells during spermatogenesis. The FSHR and LHR are the basis for the physiological functions of follicular stimulating hormone (FSH) and luteinizing hormone (LH). One study showed that male rats orally administered $AlCl_3$ at 100 mg/kg BW for 8 weeks exerted significant decrease of FSH and LH [6]. Also, male albino rat exposure to aluminum sulfate (50 mg/kg BW) for 45 consecutive days showed significant decrease in serum FSH and LH concentration [13]. But, limited data showed the effects of $AlCl_3$ on the protein and mRNA expressions of FSHR and LHR in testes.

Therefore, the effects of $AlCl_3$ exposure on the testes histological structure, the ATPase activities (Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase), and the gonadotropin receptors (measured as protein and mRNA expressions of FSHR and LHR) in the testes of rats were examined to depict the basic toxicological mechanisms of $AlCl_3$ on the testes.

Material and Methods

Rats

Eighty healthy male Wistar rats (3 weeks old) that weighed 70–95 g were allocated into four groups according to their weights (each group 20 rats). All the rats were acclimatized for 1 week, and then were provided with 0 (control group, CG), 0.4 mg/mL (low-dose group, LG), 0.8 mg/mL (mid-dose group, MG), and 1.6 mg/mL (high-dose group, HG) $AlCl_3$ in drinking water for 120 days, respectively. During the whole exposure duration, the water consumption was increased gradually with the increased of rat BW. And the water consumption of the individual rat averaged at 16 ± 2 mL/day for 100 g BW per day, resulting in the dose of $AlCl_3$ at 0, 64, 128, or 256 mg/kg BW for treatment groups. The dose of this experiment was determined referencing the research of Zhu et al. [14], which simulated the process of Al exposure in the environment, especially for workers who worked in an electroplating factory and Al mines or factories. Rats were housed in the biomedical research center, northeast agriculture university. The housing conditions were maintained according to the request of rats. The rats were kept in plastic cages (5 rats per cage). The size of all the cages is 470 × 300 × 150 mm, large enough for five mature rats. Rats were given the drinking water and food ad libitum. The health status of rats was daily monitored.

Sample Collection

After 120 days, the rats were sacrificed under light ether anesthesia, and the testes were collected from each rat. The testes were used to observe the testes morphology and examine the activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase and the protein and mRNA expressions of FSHR and LHR.

Observation of Testes Microstructure

The 10% neutral buffered formalin solution-fixed testes were embedded in paraffin; sections with a thickness of 5–6 μm were sliced from the paraffin-embedded blocks and stained with hematoxylin and eosin (H&E) stain [15]. Then, the sections were viewed and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached photographic machine (Olympus E-330, Olympus Optical Co. Ltd., Japan).

Observation of Testes Ultrastructure

The testes samples were fixed in 2.5% glutaraldehyde solution for a week. The samples were embedded in Spurr's resin by using Leica/LKB Embedding Capsules Easy Molds. Embedded samples were sectioned to a thickness of 600 Å and stained with lead staining solution and analyzed with a transmission electron microscope T-400 (Philips, Eindhoven, NL).

The Detection of ATPase Activity in the Testes

The one part of each testis was quantified 0.1 g, and the sample was processed to obtain 10 mL of 10% testes tissue homogenate. The tissue homogenate had been centrifuged at 3000 rpm for 10 min. The supernatant of the tissue homogenate was collected and used to detect the activity of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase using ^{125}I radioimmunoassay (RIA) kits (New Bay Biological Technology Co., Ltd., Tianjin, China) [12]. The experiment procedure was followed by kit introduction.

Table 1 The primer sequence of the FSHR, LHR, and β-actin

Gene	Number	Upstream and downstream primer sequence	Primer length (bp)	Product length (bp)
FSHR	NM_199237	F:5′-GTCCTCATCAAGCGACACCA-3′ R:5′-GGAGGCAGAAATGGCAAAGA-3′	23 23	103
LHR	NM_012978	F:5′-CTATCTCCCTGTCAAAGTAA-3′ R:5′-CCATTTCCATACAGTTTTAG-3′	22 22	384
β-actin	NM_031144	F:5′-CGGGACCTGACAGACTACCT-3′ R:5′-AGACAGCACTGTGTTGGCATAG-3′	20 20	328

Detection of FSHR and LHR Protein Expression

The testes sample was fixed and embedded by paraffin, then stained with FSHR and LHR immunohistochemistry kits (Beijing Biosynthesis Biotechnology Co. LTD, Beijing, China/Wuhan Biosynthesis Biotechnology Co. LTD., Wuhan, China). Sections were observed under a microscope (model: BA400, Motic, German). The positive gray value was measured by Motic image 3.2 micrograph analysis software (Motic, German), which was applied to quantify the nuclear FSHR and LHR contents. The positive gray value was negatively correlated with the protein expression of FSHR and LHR.

Determination of FSHR and LHR mRNA Expression

The expressions of the FSHR and LHR mRNA were detected in the testes by quantitative real-time PCR (QRT-PCR) with SYBR Premix Ex Taq kit (Takara, Shiga, Japan). The primers of FSHR and LHR were designed by Premier Premier 5.0 and OLIGO 6.0 Software and were synthesized by Sangon biotech (Shanghai, China) (Table 1). The relative expression of target genes was determined by the $2^{-\Delta\Delta CT}$ method [16]. The detail process was conducted according to Sun et al. [17].

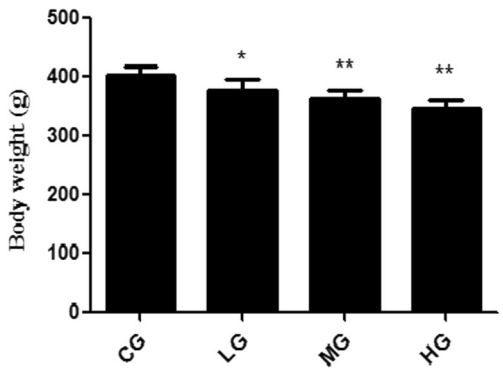


Fig. 1 Effects of $AlCl_3$ on the BW of rats. The rats were orally exposed to 0 (control group, CG), 0.4 mg/mL (low-dose group, LG), 0.8 mg/mL (mid-dose group, MG), and 1.6 mg/mL (high-dose group, HG) $AlCl_3$ in drinking water for 120 days. Data are means ± SD (n = 20 per group). *$p < 0.05$, **$p < 0.01$ versus CG

Statistical Analysis

Statistical analyses were done using SPSS 22.0 package programmer (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance followed by LSD test. Data are shown as least square means and standard deviation (SD, bar on the top of each column). The p values of less than 0.05 were considered significant, and p values of less than 0.01 were considered markedly significant.

Results

BW of Rats

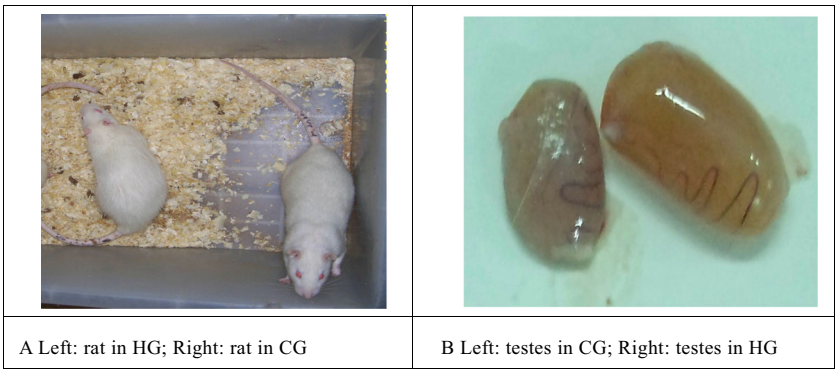
As shown in Fig. 1, the BW of rats was decreased gradually with the increase of $AlCl_3$ dose, and the BW of rats in LG, MG, and HE was significantly lower than that in CG on day 120 ($p < 0.05$; $p < 0.01$).

Clinical Symptoms

The control rats had nice sprite, normal eating and drinking skills, light clothing hair, and normal dung. With the increase of the $AlCl_3$ dose, the rats exerted bad, anorexia, loose clothing hair. The rats walk stumble and the tail of the rats got pale. The response to the outside stimulation turned weakness and the condition would get worse with the increase of $AlCl_3$ dose (Fig. 2a). In MG and HG, the rats exerted polypnea and anorexia. The rats in HG were dissected and found that the testes represented congestion, bleed, edema, and wither compared with CG (Fig. 2b).

Microstructure of Testes

The testes microstructure image were examined using paraffin section that was stained routinely with H&E (Fig. 3). The testes microstructural features in the CG revealed intact



A Left: rat in HG; Right: rat in CG

B Left: testes in CG; Right: testes in HG

Fig. 2 Effects of $AlCl_3$ on the clinical symptoms of rats. CG control group, HG high-dose group

morphology and structure of spermatogenic epithelial cells, without shedding of cells in lumen, and a large number of sperms (Fig. 3a). We found that testicular stroma slightly expanded, the number of spermatogenic cells decreased, the number of sperm decreased in the LG (Fig. 3b); testicular interstitial eosinophilic enhanced, spermatogenic cells fall into the lumen, the number of sperm significantly decreased in the MG (Fig. 3c); testicular stroma significantly expanded, the seminiferous tubule lumen was narrow, fewer sperm in the HG (Fig. 3d).

Ultrastructure of Testes

The testes ultrastructural features was observed using transmission electron microscope (Fig. 4). The testes ultrastructural features in the CG revealed intact cell membrane, dispersed chromatin, and normal morphology of mitochondria (Fig. 4a). In the treatment groups, we found that cell disintegration and incomplete cell membrane, lots of foam-like structure, mitochondrial swelling, irregular nuclear envelope (Fig. 4b–d).

The Activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase in Testes

The activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase in testes were gradually decreased with the increase of $AlCl_3$ (Fig. 5). The activity of Mg^{2+}-ATPase in MG ($p < 0.05$) and HG ($p < 0.01$) was significantly lower than those in CG. The activities of Na^+-K^+-ATPase and Ca^{2+}-ATPase in HG ($p < 0.05$) were significantly lower than those in CG.

The Protein Expressions of FSHR and LHR in Testes

The protein expressions of FSHR and LHR were showed in the four groups (Figs. 6 and 7). The positive gray value of FSHR and LHR was increased in $AlCl_3$-treated rat testes. The positive gray values of FSHR and LHR in MG ($p < 0.05$) and HG ($p < 0.01$) were significantly higher than those in CG (Fig.~8). The positive gray values of FSHR and LHR in testes were negatively correlated with the protein

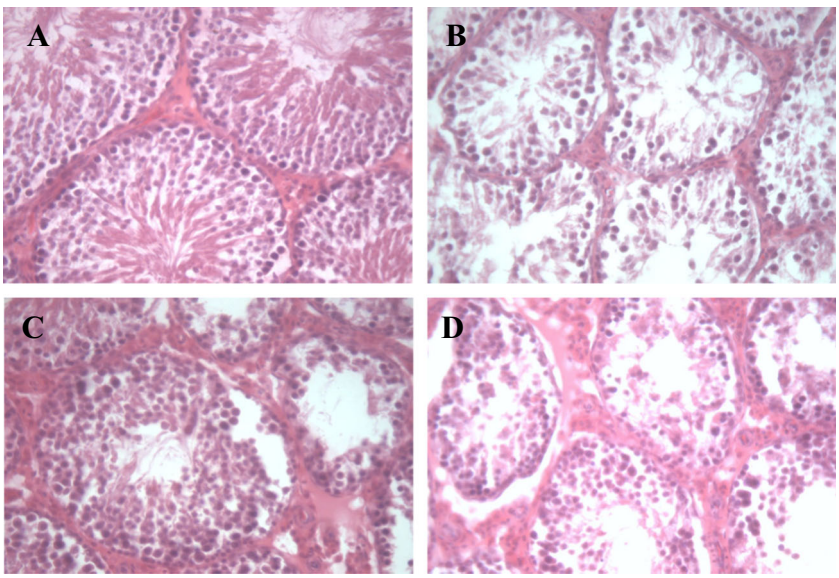


Fig. 3 Effects of $AlCl_3$ on the testes microstructure of rats. The microstructure of testes was measured by H&E stain. **a** CG (control group). **b** LG (low-dose group). **c** MG (mid-dose group). **d** HG (high-dose group). (× 400)

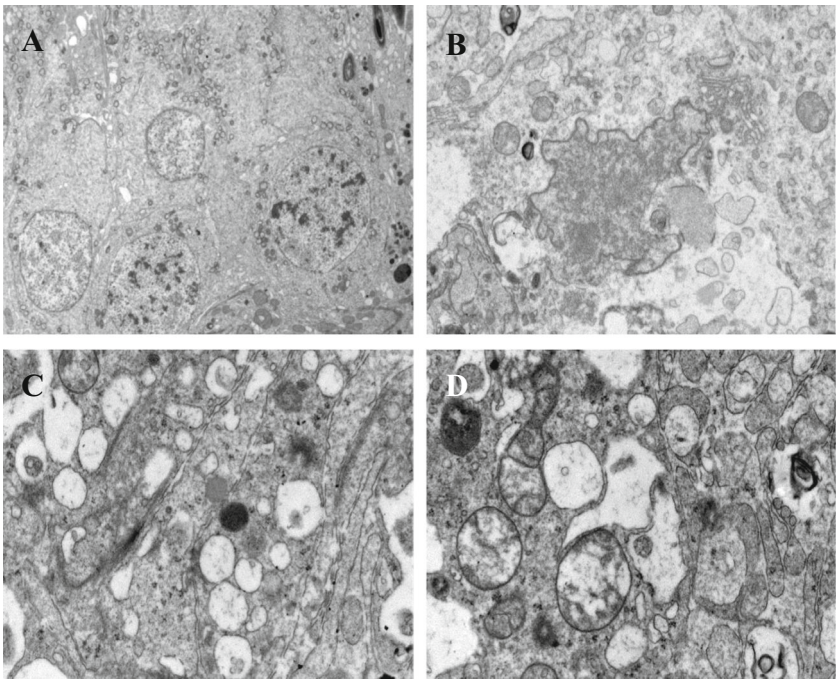


Fig. 4 Effects of $AlCl_3$ on the testes ultrastructure of rats. The ultrastructure of testes was observed by transmission electron microscope. **a** Testes tissue of rats in CG (control group) (×2550). Testes tissue of rats in HG (high-dose group), respectively, at × 9000 (**b**), × 16,500 (**c**), and × 16,500 (**d**)

expressions of FSHR and LHR. Therefore, the protein expressions of FSHR and LHR were decreased gradually with the increase dose of $AlCl_3$. The protein expressions of FSHR in MG and HG were significantly lower than in CG ($p < 0.01$).

The mRNA Expressions of FSHR and LHR in Testes

The FSHR and LHR mRNA expressions were gradually decreased in $AlCl_3$-treated rats compared with CG (Fig.~9). The FSHR and LHR mRNA expressions in MG and HG were markedly lower than those in CG ($p < 0.01$). The FSHR and LHR mRNA expressions in testes were positively correlated with the protein expressions of FSHR and LHR. Therefore, FSHR and LHR mRNA expressions were gradually decreased in $AlCl_3$-treated rats compared with CG. The FSHR and LHR mRNA expressions in MG and HG were markedly lower than in CG ($p < 0.01$).

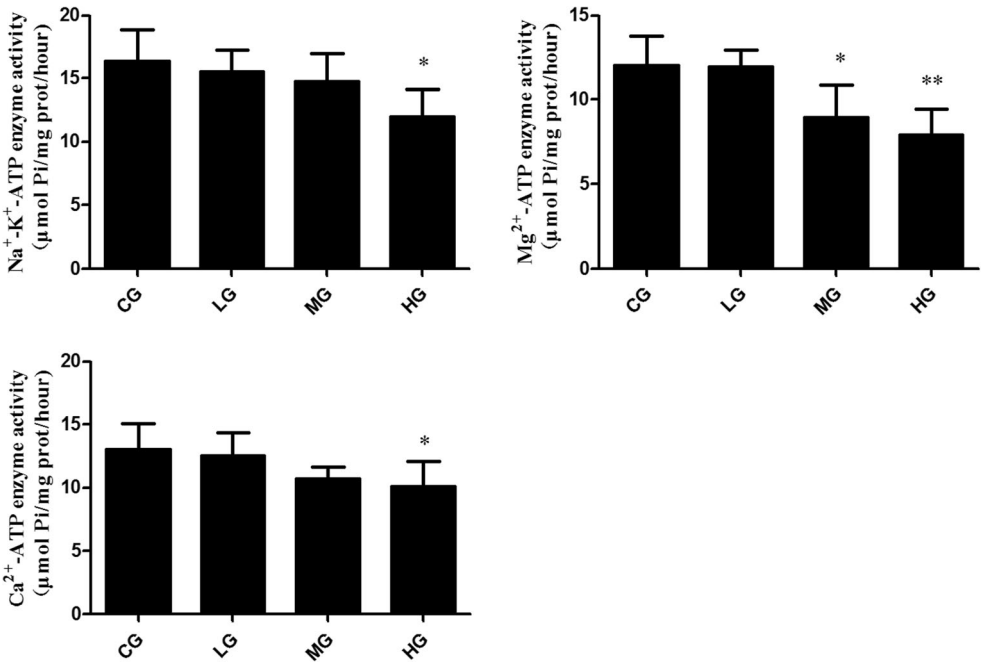


Fig. 5 Effects of $AlCl_3$ on the activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase in testes. The activities of Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase were detected by ^{125}I radioimmunoassay (RIA) kits. *CG* control group, *LG* low-dose group, *MG* mid-dose group, *HG* high-dose group. Data are means ± SD ($n = 20$ per group). *$p < 0.05$, **$p < 0.01$ versus CG

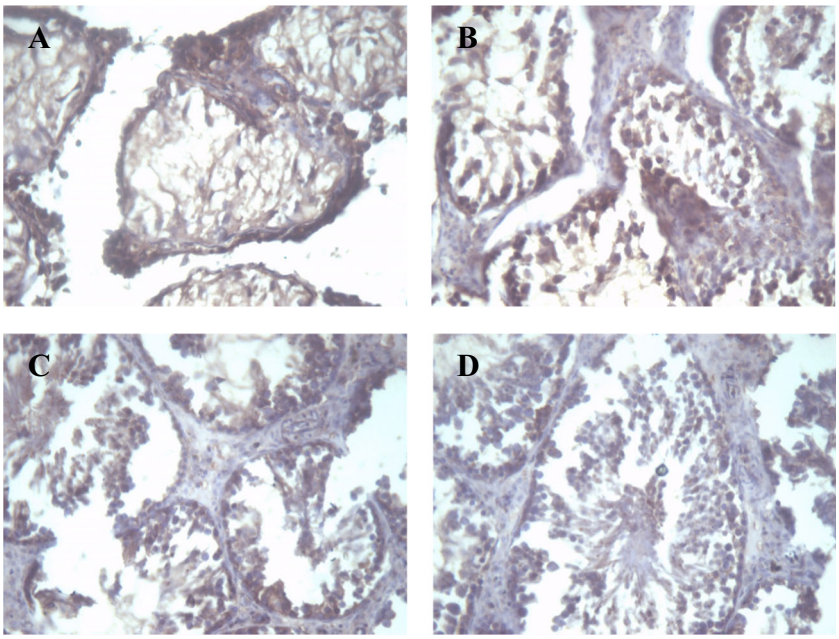


Fig. 6 Effects of $AlCl_3$ on the protein expression of FSHR in testes. The protein expression of FSHR was detected by immunohistochemistry kits. *CG* control group (**a**), *LG* low-dose group (**b**), *MG* mid-dose group (**c**), *HG* high-dose group (**d**) × 400

Discussion

Al is well-known water contaminant. Atmospheric acidification and bauxite mine over-exploitation are causing a progressively massive export of Al from mountains to surface waters, putting animals and humans in contact with absorbable cationic Al [18]. In this experiment, Wistar rats were orally administered of $AlCl_3$ in drinking water, which simulated the process of Al exposure in the environment. Furthermore, orally administered $AlCl_3$ in drinking water can reduce the stress response of rats caused by intragastric administration or intraperitoneal injection of $AlCl_3$. Thus, oral delivery of aluminum to rats was chosen in this study.

In this study, no deaths were observed in $AlCl_3$-treated rats. But the rats exerted bad, anorexia, loose clothing hair. Compared with CG, congestion, bleed, edema, and wither were observed in the testes of HG. Furthermore, $AlCl_3$ exposure damaged the cell nucleus, mitochondria, and cell membrane, disordered testicular stroma, and decreased the number of sperm, which agreed with previous study [10]. These results implied that $AlCl_3$ exposure disrupted the structure and inhibited the development of the testes, which preliminarily confirmed that the rat model with $AlCl_3$ exposure was built.

Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase were the markers of the cell impairment during toxic exposure

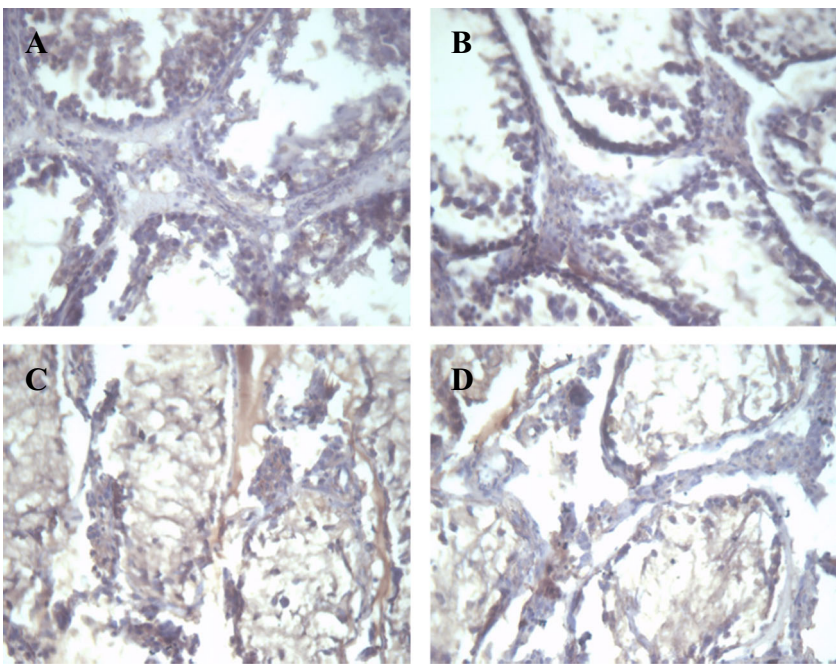


Fig. 7 Effects of $AlCl_3$ on the protein expression of LHR in testes. The protein expression of LHR was detected by immunohistochemistry kits. *CG* control group (**a**), *LG* low-dose group (**b**), *MG* mid-dose group (**c**), *HG* high-dose group (**d**) × 400

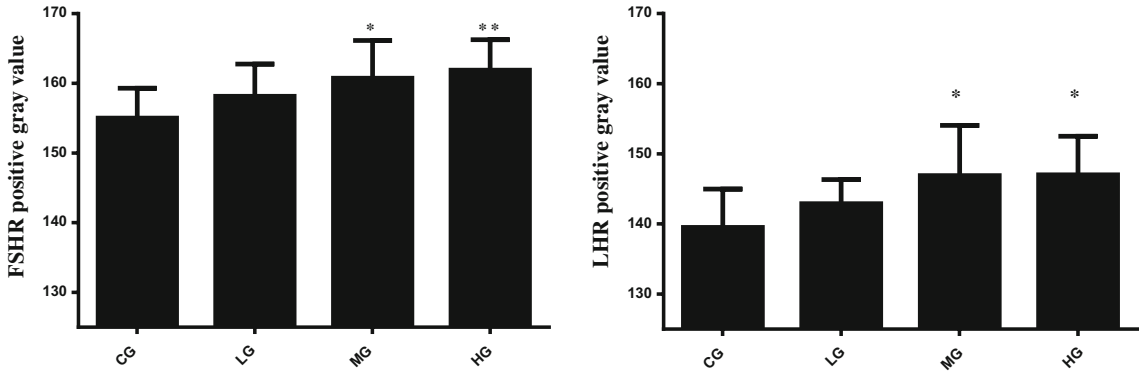


Fig. 8 The positive gray value of FSHR and LHR in testes. The positive gray value was negatively correlated with the protein expressions of FSHR and LHR. *CG* control group, *LG* low-dose group, *MG* mid-dose group, *HG* high-dose group. *$p < 0.05$, **$p < 0.01$ versus CG

[19]. In this experiment, the activities of the Na^+-K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase ATPase were gradually decreased with $AlCl_3$ exposure, indicating that $AlCl_3$ exposure induced the intracellular accumulation of Na^+ and Ca^{2+}, leading to the cytotoxic effect and impairment of the cells in the testes. Al can directly interfere with –SH groups of enzyme at the active site, thus prevents the -SH groups from functioning in certain chemical reactions [20]. The –SH groups are involved in the maintenance of the membrane-bound Na^+-K^+ATPase oligomeric structure [21]. It indicates that Al lowers the activities of Na^+-K^+-ATPase, Ca^{2+}-ATPases, and Mg^{2+}-ATPases by the reduction of –SH groups. In addition, Al perturbed the structure and functions of cell membranes through regulating apical Cl^- secretion and ATPase inactivation in class of lipids [22, 23]. Na^+-K^+-ATPase enzyme presents the cell membrane of all the animals. Thus, $AlCl_3$ can decrease the activity of Na^+-K^+-ATPase by disruption of the membrane. Calcium (Ca) is actively transported into intracellular organelles and out of the cytoplasm by Ca^{2+}/Mg^{2+}-ATPases located in the endoplasmic reticulum and plasma membranes [24]. A recent investigation showed that disintegration of endoplasmic reticulum was observed when young gerbils were injected intraperitoneally with $AlCl_3$ for 5 weeks [25]. Thus, the decrease of Ca^{2+}/Mg^{2+}-ATPases attributed to the disintegration of endoplasmic reticulum induced by Al. Al binds to the plasma membrane phospholipids, alters the lipid protein interaction, and modifies the activities of the transporters [26]. Al^{3+} accelerates Ca^{2+} release from mitochondria and strongly inhibits Ca^{2+}-ATPase activity [27]. These results indicate that $AlCl_3$ can induce ionic disorders by altering ATPases activity and contribute to testes dysfunction during intoxication.

FSH and LH, synthesized and secreted by the pituitary, regulate spermatogenesis through sperms and testosterone during adulthood. Sun et al. [17] found that the concentration of LH decreased in all $AlCl_3$-treated groups. In addition, $AlCl_3$ decreased the zinc (Zn) concentration in rats [5]. And Zn-deficient animals, chiefly males, have been shown to have lower concentrations of FSH and LH [28]. Thus, $AlCl_3$ might inhibit the secretion of FSH and LH through reducing Zn concentration. The physiological functions of FSH and LH are mediated by specific receptors—FSHR and LHR. In this experiment, the mRNA and protein expressions of FSHR and LHR in the testes decreased in the rats with $AlCl_3$ exposure. Rawi and Seif Al Nassr [13] found that the concentration of LH was decreased, but there were no significant changes in

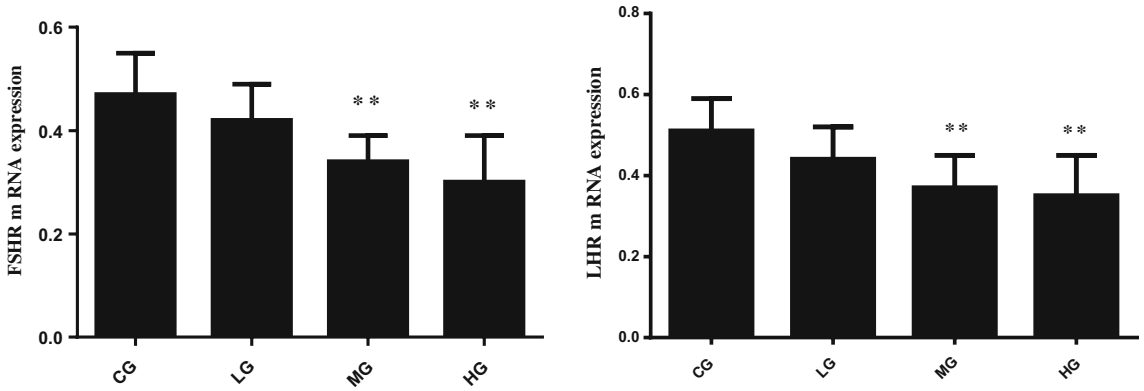


Fig. 9 Effects of $AlCl_3$ on FSHR and LHR mRNA expressions in testes. The FSHR and LHR mRNA expressions in testes were detected by QRT-PCR. *CG* control group, *LG* low-dose group, *MG* mid-dose group, *HG* high-dose group. Data are means ± SD ($n = 20$ per group). **$p < 0.01$ versus CG

FSH concentration in $AlCl_3$-treated male rats. Thus, we deduced that the lower level of LH was a reason for the degeneration of LHR with $AlCl_3$ exposure. So, $AlCl_3$ might inhibit not only the secretion of LH, but also the expressions of FSHR and LHR. However, the FSH concentration is not related to the protein and mRNA expressions of FSHR and LHR, which needs to be explored.

Al toxicity elicits a dysfunctional tricarboxylic acid cycle and impedes ATP production [29]. Study showed that mitochondrial ATP production was suppressed by generating ROS via transduction [30]. Studies showed that Al accumulation induced testicular oxidative stress [31, 32]. Thus, we deduced that Al may also suppress mitochondrial ATP production by transduction of generating ROS. Then, the lower energy will block the mRNA and protein expressions of FSHR and LHR in the testes. Furthermore, the lower activities of Na^+-K^+ATPase, Ca^{2+}-ATPases, and Mg^{2+}-ATPases induced by $AlCl_3$ lead to the intracellular accumulation of Na^+ and Ca^{2+}, inducing cytotoxic effect and impairment of the cells in the testes (sertoli cells and leydig cells). So, the lower mRNA and protein expressions of FSHR and LHR may attribute to the lower activity of Na^+-K^+-ATPase, Ca^{2+}-ATPases, and Mg^{2+}-ATPases. The FSHR and LHR expressed on sertoli cells and leydig cells in the testes. Leydig cell hyperplasia and a destruction of inter-sertoli cell tight junctions were observed [5]. These results indicate that $AlCl_3$ exposure to the decrease of protein expression of FSHR and LHR may cause the dysfunction of testes.

In conclusion, $AlCl_3$ exposure damaged the structure of the test, inhibited the ATPase enzymes activities and protein and mRNA expressions of gonadotropin receptors in the testes of rats, which indicated that $AlCl_3$ disrupted the reproductive regulation of testes.

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Compliance with Ethical Standards The experimental protocol was approved by the Ethics Committee on the Use and Care of Animals, Northeast Agricultural University, China.

Conflict of Interest The authors declare that they have no conflict of interest.

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