Original Article

Toxic Effects of Atrazine on Reproductive System of Male Rats*

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Abstract

Objective This study was designed to evaluate the toxic effects of Atrazine (ATZ) on the reproductive system of male rats.

Methods Male Sprague-Dawley rats were exposed to ATZ by gavage at dosages of 0, 38.5, 77, and 154 mg/kg bw/day for 30 d. The toxic effects of ATZ to rats were assessed through histopathologcal observation, spermatozoa quality evaluation, testicular marker enzyme indicators, antioxidant capacity and reproductive hormone levels.

Results Significant adverse effects on reproductive system were observed in rats exposed to ATZ at different dosages compared with 0 mg/kg group, including an irregular and disordered arrangement of the seminiferous epithelium in 154 mg/kg group; a decreased spermatozoa number and an increased spermatozoa abnormality rate in 77 and 154 mg/kg groups; decreased levels of acid phosphatase (ACP), alkaline phosphatase (AKP), lactic dehydrogenase (LDH), and succinate dehydrogenase (SDH) with the increasing of ATZ concentration; a decreased level of total antioxidant capacity (TAC) in a dose-dependent manner, and a decreased reduced glutathione (GSH) level and an increased malondialdehyde (MDA) content in 154 mg/kg group; and decreased serum levels of testosterone (T) and inhibin-B (INH-B) and an increased serum level of follicle stimulating hormone (FSH) in 77 and 154 mg/kg groups, and an increased serum level of luteinizing hormone (LH) in 154 mg/kg group.

Conclusion These results suggested that relatively high doses of ATZ could exert reproductive toxicity of male rats.

Key words: Atrazine; Reproductive toxicity; Oxidative stress; Endocrine disrupter

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INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATZ), a chloro-s-trazine herbicide, is used extensively worldwide for broadleaf and grassy weed control in corn, sorghum, sugarcane, cotton, and pineapple crops and landscape vegetation[1], so that ATZ and its metabolites are widely persistent in water and are mostly found in soil especially in farming seasons[2-4]. Despite ATZ has been banned in European Union and been restricted in other countries, it is still being used in large quantities worldwide up to now. It is one of the most widely used agricultural pesticides in United States[5], and its application in Asian countries has been growing. Therefore, humans and wildlife are at risk for exposure to ATZ.

In recent decades, there has been an increasing

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concern on clarifying the toxicological mechanisms of environmental chemicals to cause alterations in the reproductive system of humans and mammals. A number of reports have suggested that ATZ might have adverse effects on the reproductive function[6-13]. It has been shown that ATZ could reduce pituitary, prostate and seminal vesicle weights in rats, associating with decreased spermatozoa count, viability and motility[8-10], and cause infertility in men living in agricultural areas[11]. It has also been reported that ATZ could induce endocrine disruption and consequently interfere with physiological functions of various hormones. It was demonstrated that ATZ exposure from postnatal day (pnd) 21 to 53 had caused a significant decrease in serum and testicular T levels after administered at doses of 100-200 mg/kg bw/day in male Wistar rats[9]. Consistent with these observations, Friedmann demonstrated that ATZ in the dose of 50 mg/kg bw/day had significantly reduced the serum and testicular T levels, both in acute toxicity test (from pnd 46 to 48) and chronic toxicity test (from pnd 22 to 48), in juvenile Sprague-Dawley male rats by gavage[12]. A recent study demonstrated that the serum levels of testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), and inhibin-B (INH-B) had decreased by 85% after 48 d exposure to high dose (300 mg/kg bw/day) of ATZ in adult male Wistar rats[13]. However, there is still little knowledge on the mechanisms of ATZ-induced detrimental impacts on spermatogenesis cells, and the effects of ATZ on serum levels of T, FSH, LT, and INH-B, especially the intrinsic correlation among these hormones, are not yet well clear.

In this study, the toxic effects of a 30-d exposure to ATZ on the reproductive system of male Sprague-Dawley rats were evaluated by a panel of assays, including histopathological observation of testes, measurements of spermatozoa count and spermatozoa abnormality rate, and detections of testicular marker enzymes, antioxidative capacity and the reproductive hormone levels in serum, to further investigate the mechanisms of the reproductive toxicity of ATZ in male rats and provide insights and perspectives for the potential harm of ATZ to human health.

MATERIALS AND METHODS

Chemicals

ATZ ($C_8H_{14}ClN_5$, CAS: 1912-24-9, ≥98% purity) was from Kesai Chemical Limited Company (Jinan, China). T, FSH, LH, and INH-B enzyme linked immune sorbent assay (ELISA) kits were from R&D systems (USA). Reagent kits of total protein (TP), alkaline phosphatase (AKP), acid phosphatase (ACP), lactic dehydrogenase (LDH), succinate dehydrogenase (SDH), total antioxidant capacity (TAC), reduced glutathione (GSH), and malondialdehyde (MDA) were from Nanjing Jiancheng Bioengineering Institute (China).

Animals and Treatments

Forty male Sprague-Dawley rats of Specific Pathogen Free (SPF) grade (aged 4 weeks and weighted 80±10 g) were purchased from Dashuo Laboratory Animal Reproduction Center in Jianyang, China [Certificate number: SCNK (CHUAN) 2008-29]. Animals were housed in polycarbonate cages in an environmentally controlled room (temperature 20 to 24 °C and 12 h light/12 h dark). Food and water were provided *ad libitum*.

After one-week acclimation, the rats were randomly divided into 4 groups, i.e. a control group and 3 treatment groups. ATZ was dissolved in corn oil and given to the rats by gavage at doses of 0 mg/kg bw/day (corn oil control), 38.5 mg/kg bw/day (ATZ-L), 77 mg/kg bw/day (ATZ-M) and 154 mg/kg bw/day (ATZ-H) respectively. The gavage volume was 5 mL/kg bw and the amount of gavage was adjusted every three days according to the varied weight of each rat. The total exposure duration was 30 consecutive days, and the solution of ATZ in corn oil was prepared each week. At the end of the experiment, the rats were sacrificed, and their testes, epididymis and accessory glands (seminal vesicles and prostate) were carefully dissected out and weighed.

In this study, all the experiments conducted in animals were in accordance with the guidance of ethical committee for research on laboratory animals of Sichuan University.

Histopathological Evaluation of Testis

The removed testes were fixed in Bouin's fixative as described by Zhen et al.[14]. After 24 h, testes were washed for three times and maintained in 70% ethanol. Samples were then embedded in paraffin and sectioned with rotary microtome (GMbh, Germany). The tissue sections of the testes were then stained with hematoxylin and eosin (H&E), and observed with an optical microscope (AX70, Olympus, Japan) with blind manner.

Spermatozoa Count and Spermatozoa Abnormality Rate Assay

One epididymis of each rat was isolated and cleared of adhering tissues. The epididymis was chopped into small pieces (about 1 mm^3) and incubated in 10 mL of Ham's F-12 medium at 37 °C for 1 h as described by Chitra et al.[15]. Then, 1 mL of the above solution was diluted with 9 mL Ham's F-12 medium, and the diluted solution was transferred into the chamber of the haemocytometer, and spermatozoa number was counted. Microscopic examination of seminal smears stained with eosin was carried out to determine the spermatozoa abnormality according to the method as described by Yokoi et al.[16].

Testicular Marker Enzyme Assay

A part of testis after ice water washing and minced with scissors was homogenized in 9 volumes of 0.9% saline solution and centrifuged at 15 000 g at 4 °C for 15 min as described by Kalla et al.[17]. The supernatant was used for the assay of TP, ACP, AKP, LDH, and SDH, based on the methodology of assay kits.

Measurement of the Testicular Antioxidant Capacity

To evaluate the toxic effects of ATZ on antioxidant capacity, levels of TAC, GSH, and MDA were respectively measured with spectrophotometric methhds, i.e. the assessment of TAC was carried out based on the ferric reduction antioxidant power (FRAP) assay as described by Benzie et al.[18]; the level of GSH was determined according to the method as described by Sedlack and Lindsay[19]; the MDA content of the testes was measured by using the thiobarbituric acid (TBA) reaction as described by Lowry et al.[20].

Serum Hormone Analysis

The blood sampled by eyeball extirpating was collected in 15 mL centrifuge tubes and allowed to clot overnight at 4 °C. These blood samples were centrifuged at 10 000 g at 4 °C for 10 min and sera were collected. Then serum T, LH, FSH, and INH-B levels were measured by ELISA kits, according to the manufacturer's protocol.

Statistical Analyses

Data were presented as Means(x)±standard deviation(s). Statistical analyses were performed by using one-way analysis of variance (ANOVA) followed by Dunnett's t-test in SPSS for windows version 17.0 (SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at $P<0.05$ compared with the control group.

RESULTS

Toxic Effect of ATZ on Body Weight and Organ Weights

As shown in Table 1, after 30-d ATZ exposure, the weights of body and testes in ATZ-H group decreased significantly compared with the control group ($P<0.05$). There was no significant difference in epididymis weight, seminal vesicle weight and prostate weight between ATZ treated groups and the control group.

Toxic Effect of ATZ on Change of Histopathology of Testis

Figure 1 showes the morphological changes in the testicular tissue in the control group and the treated groups. A compact and regular arrangement of cells in seminiferous tubules was showed in the control group, and nearly all stages of spermatogenesis were found in a cross-section of the seminiferous tubules (Figure 1A). Microscopic examination of the testes of rats treated with low dose ATZ revealed normal histological structure of most seminiferous tubules with normal spermatogenesis (Figure 1B). The ATZ-H group showed a disruption of normal spermatogenic cell

Table 1. Toxic Effects of ATZ on Body Weight and Organ Weights (n=10, x±s)

Groups	Body (g)	Testes (g)	Epididymis (g)	Seminal Vesicle (g)	Prostate (g)
Control	209.83±25.21	2.73±0.28	0.51±0.08	0.42±0.17	0.15±0.04
ATZ-L	195.26±30.38	2.72±0.36	0.51±0.09	0.33±0.24	0.14±0.05
ATZ-M	194.06±26.28	2.66±0.16	0.46±0.08	0.36±0.17	0.16±0.05
ATZ-H	172.96±22.97*	2.42±0.14*	0.43±0.09	0.30±0.22	0.14±0.06

Note. *$P<0.05$, compared with the control group.

organization with visible holes among the cells in the tubules, and the total number of germ cells inside the tubules decreased dramatically and the spermatocytes were connected to the lumen, indicating cell disorganization (Figure 1D). In ATZ-M group, the damage of the seminiferous tubules was considerably less severe: spermatogenesis was still present, but the number of cells decreased per unit of area compared with the control group (Figure 1C).

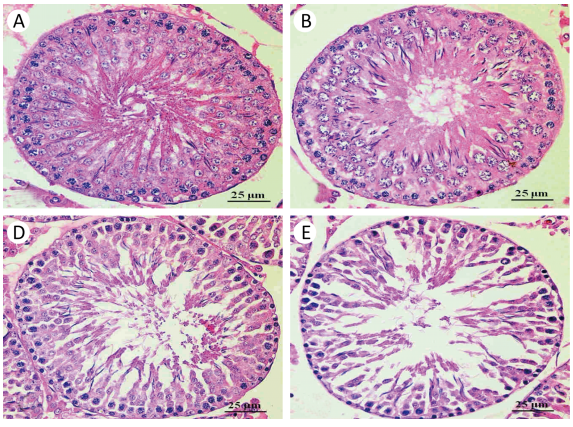

Figure 1. Histopathologic changes in testicular tissue after ATZ exposure for 30 d. The testicular tissue was sectioned with a thickness of 5 μm and stained with H&E. Magnification, 400X. (A) Control, (B) ATZ-L, (C) ATZ-M, (D) ATZ-H.

Toxic Effects of ATZ on Decrease of Spermatozoa Number and Increase of Spermatozoa Abnormality Rate

The data of spermatozoa count and spermatozoa abnormality rate assay were presented in Table 2. Compared with the control group, the spermatozoa count of epididymis of ATZ-M and ATZ-H groups decreased dramatically (P<0.01), while the total abnormal spermatozoa percentage increased significantly in a dose-dependent manner. More specifically, the percentage of amorphous and coiled-tailed spermatozoa increased significantly in ATZ-H group (P<0.01), and the percentage of double-headed and hookless spermatozoa increased significantly in ATZ-M and ATZ-H groups (P<0.01).

Toxic Effects of ATZ on Decrease of Activity of Testicular Marker Enzymes

The toxic effects of ATZ on the activity of testicular marker enzymes were presented in Table 3. The results indicated that the activity of four enzymes decreased significantly with the increase of ATZ concentration, compared with the control group (P<0.05, P<0.01, respectively).

Table 2. Toxic Effects of ATZ on Spermatozoa Characteristics in Rats (n=10, x±s)

Groups	Count (10^7/mL)	Abnormality (%)				
		Amorphous	Double-headed	Hookless	Coiled-tailed	Total
Control	9.56±2.26	2.76±0.83	0.39±0.23	1.54±0.41	0.33±0.17	5.01±0.81
ATZ-L	8.74±2.54	3.09±0.58	0.35±0.13	1.88±0.55	0.35±0.22	5.66±0.95
ATZ-M	6.86±1.10**	3.43±1.15	0.56±0.14**	2.17±0.52**	0.47±0.14	6.63±1.58**
ATZ-H	6.63±1.18**	5.61±1.68**	0.84±1.51**	3.31±0.82**	0.76±0.19**	10.53±2.54**

Note. **P<0.01, compared with the control group.

Table 3. Toxic Effects of ATZ on Activity of Testicular Marker Enzymes in Rats (n=10, x±s)

Groups	ACP (U/g pro)	AKP (U/g pro)	LDH (U/g pro)	SDH (U/mg pro)
Control	28.38±1.68	13.84±2.10	2 118.40±112.45	16.76±2.72
ATZ-L	28.69±2.60	12.85±2.47	1 995.93±113.58	15.97±2.07
ATZ-M	26.26±3.76	10.22±1.50**	2 089.24±174.34	13.73±2.36**
ATZ-H	24.82±2.32*	9.98±1.35**	1 833.50±184.25**	13.51±1.74**

Note. *P<0.05 and **P<0.01, compared with the control group.

Toxic Effects of ATZ on Decrease of Testicular Antioxidant Capacity

As shown in Table 4, biochemical analyses indicated that the level of TAC decreased significantly depending on dose (P<0.05, P<0.01, respectively), and GSH level decreased significantly in ATZ-H group (P<0.05), while the MDA content of the testes in ATZ-H group increased significantly (P<0.05), in comparison with the control group.

Toxic Effects of ATZ on Serum Hormone Levels

The toxic effects of ATZ on serum hormone levels are shown in Figure 2. The serum T and INH-B levels dropped significantly in ATZ-M (P<0.05) and ATZ-H groups (P<0.01). In contrast, the serum FSH level in ATZ-M and ATZ-H groups rose significantly (P<0.05, P<0.01, respectively), and the serum LH level in ATZ-H group also increased remarkably (P<0.05).

Table 4. Toxic Effects of ATZ on Testicular Antioxidant Capacity in Rats (n=10, $x\pm s$)

Groups	TAC (U/mg pro)	GSH (U/mg pro)	MDA (nmol/mg pro)
Control	11.79±1.57	60.55±6.54	1.98±0.55
ATZ-L	9.92±1.88*	56.85±6.76	2.01±0.55
ATZ-M	7.52±1.15**	50.09±9.38	1.95±0.73
ATZ-H	6.01±2.12**	36.55±7.31*	2.52±0.63*

Note. *P<0.05 and **P<0.01, compared with the control group.

DISCUSSION

It has been reported that ATZ could induce reproductive abnormalities in male rats[6-10]. A previous study demonstrated that ATZ was a low-toxic compound with the median lethal dose (LD_{50}) for rat was 3080 mg/kg[21]. Thus, the calculated doses in the present study were 1/80, 1/40, and 1/20 of the LD_{50} for the ATZ-L, ATZ-M, and ATZ-H groups, respectively.

Toxic Effect of ATZ on Histopathology of Testis

In this study, it was found that high doses of ATZ could influence the seminiferous epithelium by histopathological examination. The arrangement of cells in ATZ-M and ATZ-H groups was irregular and disordered, and intercellular connections, e.g. gap junctions, were not compact, which indicated that ATZ could pass blood-testis barrier and disturb the junction between Sertoli cells and germ cells. Furthermore, these impairments might be related to reduction of T production of Leydig cells, because it is well understood that T affects and controls the physiological functions of Sertoli cells and histological integrity[22]. Thus it might be concluded that following exposure to ATZ, Leydig cells would degenerate and decline in number per mm^2 of the interstitial tissue, and their ability for synthesis of T also decreased.

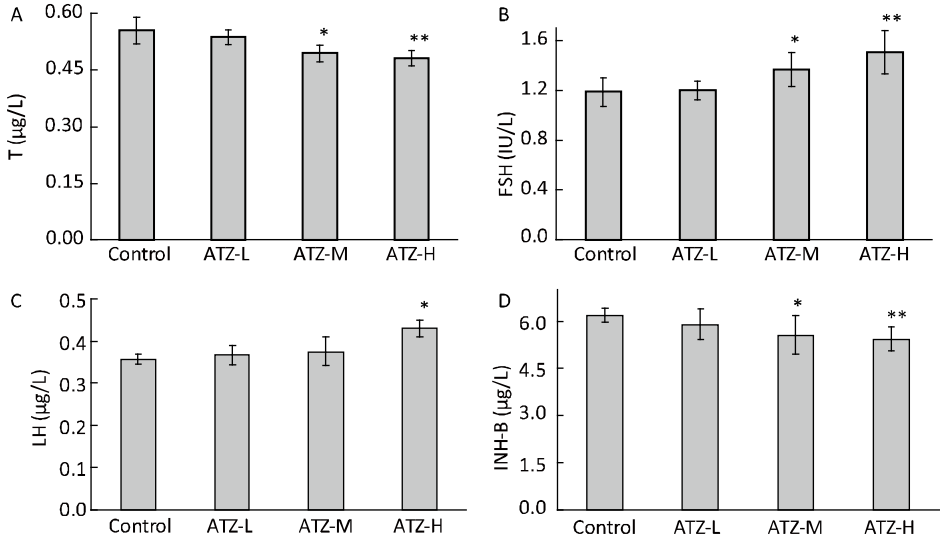

Figure 2. Effects of ATZ on the serum hormone levels (T, FSH, LH, and INH-B) of rats (n=10, $x\pm s$). *P< 0.05 and **P<0.01, compared with the control group.

Toxic Effect of ATZ on Spermatozoa Characteristics

The evaluation of the count and abnormality rate of spermatozoa is considered to be useful to detect the adverse effects on spermatogenesis quantitatively[23]. Consistent with the result of histopathological examination, the present results showed that the spermatozoa count decreased significantly, while the abnormal spermatozoa percentage increased significantly, both in a dose-dependent manner. These results suggested that high dose ATZ might penetrate the blood-testis barrier, directly affect spermatogenesis and harm specific components of the seminiferous epithelium. These effects were similar to the results that ATZ treatment caused a significant decrease in spermatozoa count and motility of rats reported by Kniewald and Dehkhargani et al.[8,24].

Toxic Effect of ATZ on Activity of Testicular Marker Enzymes

Testes are the most important organs in male reproductive system, which are involved in the functions of spermatogenesis and T secretion. In male animals, many kinds of enzymes are closely correlated with the functions of testes, and their activity keeping germ cell growing normally is the very important endpoint to assess reproductive toxicity[25-28]. To evaluate the toxic effects of ATZ on testes biochemical metabolism, the levels of ACP, AKP, LDH, and SDH in testes of Sprague-Dawley rats were detected in the study. In testis, ACP, mainly detected in cytoplasm of Sertoli cell, is associated with the denaturation of seminiferous epithelium and phagocytosis of Sertoli cells. AKP is associated with the division of spermatogenic cells and the transportation of nutrients to spermatogenic cells for proliferation and differentiation. In the present study, the activity of ACP and AKP in the rat testes decreased significantly after ATZ exposure, which indicated that ATZ might lead to dyszoospermia by disturbing nutrients transportation, interfering with energy metabolism, and decreasing the spermatogenic cells division in testes. LDH and SDH, widely distributed in the seminiferous tubules and germ cells, are associated with the maturation of spermatogenic cells and spermatozoa, as well as the energy metabolism of spermatozoa. The results from this experiment showed that the activity of LDH and SDH decreased significantly in ATZ-H group, which suggested that ATZ could play an important role in the maturation and energy metabolism of spermatogenic cells and spermatozoa.

Toxic Effect of ATZ on Testicular Antioxidant Capacity

Mammalian spermatozoa are redox active cells that can generat reactive oxygen species (ROS), e.g. H_2O_2[29]. Physiological concentrations of ROS are of fundamental biological importance in promoting spermatozoa maturation[30], controlling spermatozoa capacitation[31-32] and regulating signal transduction pathways[33]. However, an imbalance between the production and the scavenging of ROS can alter reproductive fitness[34-36]. Furthermore, the fertilizing ability of spermatozoa is dependent in part on the integrity and fluidity of the spermatozoa plasma membrane, which plays a pivotal role for the capacitation, acrosome reaction and sperm-oocyte fusion[37]. But in turn, the spermatozoa plasma membrane containing a characteristically high level of polyunsaturated fatty acids (PUFA) is susceptible to oxidative damage that initiates lipid peroxidation, culminating in reduction of membrane fluidity, increase of cell permeability and production of spermicidal end products[38-40].

In testis, GSH, which is mainly in Sertoli cells, acts in an antioxidative manner to protect sulfhydryl groups and PUFA in spermatozoa from oxidation and help in the detoxification and excretion of oxygen radicals during the spermatozoa maturation and storage process[41]. Thus, GSH is considered to be a useful indicator to assess antioxidant capacity. Lipid peroxides derived from PUFA decompose to form a series of more stable products, and the most common form is MDA[42]. The quantification of MDA is a convenient proxy for assessment of lipid peroxidation in biological systems. Furthermore, TAC, negatively correlated with ROS, was measured in this study. The results indicated that the 30-d exposure to ATZ could cause remarkable decreases in testicular levels of TAC and GSH, indicating severe ATZ-induced oxidative stress. The MDA analyses also demonstrated that ATZ could cause significant lipid peroxidation in testes tissues of ATZ-H group. The adverse effects obtained above might partly explain the results of the decreased spermatozoa count and the increased abnormality spermatozoa rate.

Toxic Effect of ATZ on Serum Hormone Levels

It is well understood that the spermatogenesis and the physiological functions of Sertoli cells in mammals depend largely on T production by Leydig cells in response to stimulation by FSH and LH[22,43-44].

It has reported that ATZ exposure had reduced intratesticular and circulating levels of T in pubertal and adult male rats[24,45-46]. In this study, the serum T level decreased significantly in ATZ-M and ATZ-H groups after the 30-d ATZ exposure, which indicated that ATZ exposure could lead to Leydig cells' degeneration and reduction, and the decrease of their capacity of T synthesis at the same time. Moreover it is well understood that any reduction in T level can lead to a severe reduction in INH-B synthesis of Sertoli cells[44]. Previous studies have demonstrated that INH-B is an important marker of the competence of Sertoli cells and spermatogenesis[47], and secretion of FSH and LH is regulated by T and INH-B through a negative feed-back mechanism of hypothalamic-pituitary-testes axis[44]. In this study, INH-B production of Sertoli cells of the rats was inhibited after ATZ exposure, which suggested that ATZ could firstly damage Leydig cells and Sertoli cells, and subsequently inhibit the T secretion of Leydig cells and the INH-B secretion of Sertoli cells, and then the decrease of the T and INH-B secretions result in the increase of FSH and LH secretion in a feed-back way.

According to a previous report, the pituitary gland was considered as a target tissue for ATZ, the exposure to ATZ could induce gonadal malformation and/or immaturity in male offspring[48]. Some reports also indicated that the serum levels of FSH and LH had decreased in ATZ treated rats[13,49-51], which is contrary to our results, i.e. the increase of the levels of FSH and LH. The difference might primarily due to the difference in exposure dosage, because the dosage of ATZ-H group (154 mg/kg dw/day) in this study was even lower than the dosages of low dose groups reported in those previous studies[13,49-51]. Therefore, it was implied that ATZ of relatively low dose range indirectly caused the increase of FSH and LH secretion, primarily by the negative feed-back in response to the decrease of T and INH-B; while above the range, ATZ directly compromised the hypothalamic-pituitary-testes axis to decrease the levels of FSH and LH as the major effect[52].

CONCLUSION

The results from the present study indicates that relatively high dose range of ATZ could damage the seminiferous tubule; influence the production of spermatozoa; decrease the activity of testicular marker enzymes; induce oxidative stress; and disturb the secretions of T, FSH, LH, and INH-B. It could be concluded that ATZ of relatively high dose range could induce reproductive toxicity in male rats, but the exact mechanisms need to be further investigated.

CONFLICT OF INTEREST STATEMENT

The authors declared that there was no conflict of interest.

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