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Running Title: Atrazine affects morpho-physiology and testicular functions

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ABSTRACT:
Some of the environmental toxicants acting as endocrine disruptors have been associated with health hazards in human and wildlife by modulating hormonal actions. The widely used herbicide; atrazine (ATZ) is a potent endocrine and testicular disruptor. However, studies on it remain largely inconclusive especially whether the effects are reversible or permanent. We therefore designed this study to evaluate the histological and hormonal changes associated with differential ATZ exposure. Twenty (20) adult male Wistar rats were divided into 4 groups (5 rats per group) control and three experimental groups. Control received the vehicle; the 3 groups received ATZ, 38.5, 77.0 and 154.0 mg/kg bw/day for 30 days respectively. The effects of Atrazine were assessed through histopathological observation, spermatozoa quality examination and reproductive hormone levels. Results showed that irrespective of the ATZ dose, there was significant decrease in weight, severe pathological changes in testicular tissue, decrease in the quality of semen and altered luteinizing hormone (LH) of the rats. Taken together, our findings showed that ATZ exposure could lead to poor reproductive ability in male Wistar rats.

Keywords: Atrazine, endocrine disruptor, testes, reproductive toxicity, Luteinising hormone

INTRODUCTION:

Globally, there is a growing concern among the scientific community, policy makers as well as general public about the adverse impact on health, in general, and reproductive potential, in particular, of a wide range of chemicals released in the environment as herbicides [1, 2]. Some of these environmental toxicants strongly act as endocrine disruptors with the potential to alter hormonal action within the body. One of such chemicals is Atrazine (ATZ). Most of these toxicants have been banned in some developed countries for agricultural and household purposes due to continuous revelation of their side effects [2, 3]. They are, however, still being used in developing countries because of their low cost, easy availability as well as the absence of safer and cheaper alternatives. These chemicals are entering into animal and human body through various means and their chronic exposure is associated with serious detrimental effects on the body system [4, 5].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), an active component found in herbicides commonly used in agriculture worldwide, has been considered a potent endocrine disruptor and cause adverse effects on the male genital system [5, 6, 7]. The importance of endocrine disruptors in males reflects the growing body of evidence highlighting the close relationship between these compounds and the increase in male reproductive disturbances of many vertebrates, including humans [5]. Reproductive problems linked to atrazine exposure include de-masculinization and feminization in fish, amphibians and reptiles [5], loss of ovarian germ cells [6], testicular degeneration in amphibians [8], structural disruption of testes in fish [9], crocodilians [10], birds [11], and rodents [12], reduction in sperm count and motility [13], weight reduction of the rat prostate and seminal vesicle [14], as well as delayed sexual maturation [15]. These reproductive defects have been associated with disrupted hormonal activity [16,17] and oxidative stress [18].

One of the several important unanswered questions is whether the effects of ATZ in the testis are primary or secondary to changes in relation to other segments of the male reproductive tract. More so, considering that ATZ [15] continues to be broadly used in large scale in agriculture across the world [4, 19], is easily disseminated in the environment, and causes adverse effects on male reproduction by acting as an endocrine disruptive agent [20], it is paramount to further investigate tissue and hormonal alterations induced by this herbicide.

Therefore, this study was designed to investigate effects of different doses of atrazine

on morpho-physiological and testicular functions in adult male Wistar rats.

METHODOLOGY:

Atrazine (ATZ) in the commercial product cotrazine 80 WP (purity, 80% wet table powder) was obtained from Nantong Foreign Trade Meheco Corporation (China). All other reagents were analytical grade chemicals.

A total of 20 adult male Wistar rats, weighing between 180g to 200g and acquired from the Animal House of the Department of Biochemistry, University of Ilorin, Nigeria, were used in this study. The animals were maintained at 12 hours light:12 hours dark cycle and were supplied with water and feed ad libitum. After one-week acclimatization, the rats were randomly assigned into four groups comprising of the control group (A) and 3 treatment groups (B – D) with 5 rats per group. The treated groups received ATZ comprising of the following dosage [5]: Group B: 38.5 mg/kg bw/day (low dose); Group C: 77.0 mg/kg bw/day (average dose); Group D: 154.0 mg/kg bw/day (high dose). ATZ was dissolved in the vehicle (distilled water, 1.0 mL kg-1) and orally administered to the treated groups at the dosage stated. The control group was administered the vehicle (1.0 mL kg-1 bw). The treatments lasted for 30 days. At the end of the experiment, the rats were humanely euthanized and their testes and epididymis were carefully dissected out and weighed. Blood was collected from the apex of the heart by cardiac puncture and stored in heparinized bottles. The testis was harvested and fixed for histological analysis.

Experimental procedures involving the animals and their care were conducted in conformity with International, National and Institutional guidelines for the care of laboratory animals in Biomedical Research and use of Laboratory Animals in Biomedical Research as promulgated by the University of Ilorin Ethical Review Committee.

Histo-pathological evaluation of testis: The testes were fixed in bouin's [21]. After 24 hours, the testes were washed and maintained in 70% ethanol. Samples were then embedded in paraffin and Sectioned with rotary microtome. The tissue sections of the testes were then stained with hematoxylin and eosin (H&E). The histological slides were viewed under light microscope and the photomicrographs of the desired sections were made at different magnifications with LCD camera of the microscope for further observations.

Assay of hormones: The plasma obtained from the blood was used for assay of Luteinizing hormone (LH) and Follicle stimulating hormone (FSH). These were done using the appropriate ELISA kits, according to the manufacturer's protocol.

Semen analysis: After the right caudal epididymis was excised, sperm was obtained using a modification of the method previously described [22]. The testis from each rat were carefully exposed and removed, they were trimmed free of the epididymis and adjoining tissues. From each separated epididymis, the caudal part was removed and placed in a beaker containing 1.0 ml of normal saline solution. Each section was quickly cut off with scissors and left for a few minutes to liberate its spermatozoa into the saline solution. Sperm motility, concentration and progressive motility were determined. Semen drops were placed on the slide with two drops of normal saline. The slide was covered with a cover slip and examined under microscope using X40 objective for sperm motility. Sperm count was done under the microscope using improved heamocytometer.

Sperm morphology: The sperm cells were evaluated with the aid of light microscope at X100 magnification. Caudal sperm cells were taken from the original dilution for motility and diluted 1:20 with 10% neutral formalin. Five hundred sperm cells from the sample were scored for morphological abnormalities [23]. In wet preparation using phase contrast optics, spermatozoa were categorized. In this study a spermatozoa was considered abnormal morphologically if it had one or more tail, rudimentary tail, round head and detached head, neck and middle piece defects.

Statistical analysis: The data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test using Graph pad prism version 5.0 software. Results were presented as mean ± standard error mean (SEM). Values of $p \leq 0.05$ were considered to be statistically significant.

RESULTS:

The body weights were significantly reduced ($p<0.05$) in all the treated groups compared with the control group (Table 1). Sperm count was significantly reduced in ATZ – exposed rats irrespective of the dosage, although it was more prominent in the high ATZ-treated groups. Percentage sperm motility, morphology and life-death ratio were significantly reducedin the treated groups as well, compared with the control group (Table 2).No pathological changes were detected in the testes of rats in the control group. The testes of the low dose ATZ-treated rats showed mild pathological lesions represented by the depletion of the nuclei, accumulation of fluid in the cells and cellular degeneration. For the rats exposed to the average dose of ATZ, their testes displayed moderate pathological changes represented by the destruction and degeneration of the connective layers of the leydig cells, the damage to the seminiferous tubule was moderate, spermatogenesis was still present, but the number of cells decreased per unit of area compared with the control group.

For the rats exposed to the high dose of ATZ, their testes displayed severe pathological changes, represented by tubular and cellular degeneration of the seminiferous tubule, a disruption of normal spermatogenic cell organization with visible holes among the cells inside the tubules, and the total number of germ cells inside the tubules significantly decreased and the spermatocytes were connected to the lumen indicating cell disorganization (Figure 1).

There was a significant decrease in the level of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the treated groups compared with the control (Table 3).

Likewise the LH/FSH ratios were significantly reduced in the treated groups compared with the control group (Table 3).

Table 1: Effects of Atrazine exposure on body weight in adult male rats

	Control	Experimental groups		
	A	B	C	D
Initial weight (g)	219.3 ± 1.5	222.0± 3.2	219.5 ± 1.4	218.8 ± 4.2
Final weight (g)	269.1 ± 4.0	215.2 ±7.7*	182.0 ± 5.9*	163.0 ± 8.2*
Weight change (g)	49.8± 1.7	-6.8± 2.0*	-37.5± 2.4*	-55.8± 3.2*

Data are expressed as mean ±S.E.M. (n=5). Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. A).

Table 2: Effects of Atrazine exposure on sperm parameters in adult male rats

	A	B	C	D
	Control	Experimental groups		
Sperm count (x10^6/ml)	89.20±2.20	67.83±0.70*	58.90±1.89*	53.70±1.13*
Sperm motility (%)	92.50±2.80	82.63±3.50	61.23±1.71*	50.34±2.34*
Sperm morphology (%)	93.20±3.34	78.26±2.45*	59.42±1.49*	68.19±3.45*
Sperm life/death ratio (%)	81.50±2.81*	65.05±3.56*	58.64±3.73*	47.39±4.47*

Data are expressed as mean ±S.E.M. (n=5). Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. A).

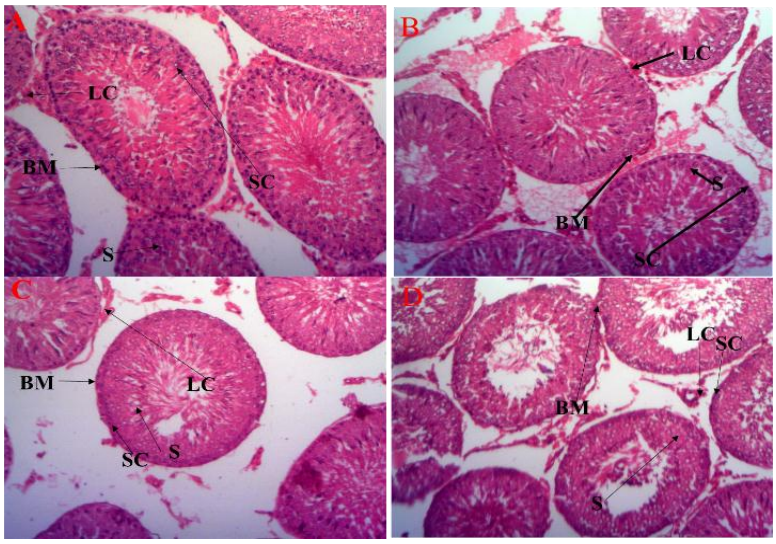

Figure 1. Histology of testicular tissue showing the effect of Atrazine exposure on the testis of adult male rats. The testes of the low dose ATZ-treated rats showed mild pathological lesions represented by the depletion of the nuclei, accumulation of fluid in the cells and cellular degeneration. For the rats exposed to the average dose of ATZ, their testes displayed moderate pathological changes represented by the destruction and degeneration of the connective layers of the leydig cells, the damage to the seminiferous tubule was moderate, spermatogenesis was still present, but the number of cells decreased per unit of area compared with the control group. For the rats exposed to the high dose of ATZ, their testes displayed severe pathological changes, represented by tubular and cellular degeneration of the seminiferous tubule, a disruption of normal spermatogenic cell organization with visible holes among the cells inside 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. (H&E paraffin stain; ×40). BM (Basement membrane), LC (Ledig cell), SC (spermatogenic cell), S (seminiferous tubule).

Table 3: Effects of Atrazine exposure on luteinizing (LH) hormone and follicle stimulating hormone (FSH) in adult male rats

	A	B	C	D
	Control	Experimental groups		
FSH (ng/ml)	9.05±1.22	6.01±0.93*	5.95±0.63*	5.39±0.75*
LH (ng/ml)	61.90±4.35	42.61±3.23*	43.04±3.01*	37.14±4.34*
FSH/LH ratio	0.156±0.002	0.140±0.001*	0.138±0.001*	0.138±0.001*

Data are expressed as mean ±S.E.M. (n=5). Data were analysed by one-way ANOVA followed by Bonferroni post hoc test. (*p<0.05 vs. A).

DISCUSSION:

The current study investigated the effects of different doses of ATZ on sperm count, motility, morphology and circulating gonadotropic hormones in male Wistar rats. Importantly, it shows the effects of ATZ exposure even in the

smallest dose used in the present study. Our results show noticeable histopathological changes and reduced sperm viability characteristics in the three experimental groups compared to the control group.

We observed a significant reduction in body weight following exposure to ATZ in all the treated groups when compared with control group.This is in line with previous reports that ATZ exposure leads to a reduction in rat body weight [24, 25]. Loss of body weight has been associated with reduction in food intake after exposure to ATZ [26]. We cannot rule out this possibility and this happens to be one of the limitations of this study as the food intake was not monitored. However, other studies have shown that irrespective of the weight change, ATZ has direct effect on the testis and androgen biosynthesis, a factor that can also interfere with the body mass [26, 27]. There was significant reduction in LH and FSH levels in the treated rats compared with the control. This is in consonance with a previous report [27]. This of course will affect the normal reproductive functions. However, the effect of ATZ on circulating gonadotropic hormones in the present study is not dose dependent. In the semen analysis, we observed significant reduction in sperm count, percentage sperm motility, morphology and life-death ratio in the treated groups compared with the control group. The observed alterations in the sperm characteristics are in line with previous reports [8, 15]. Furthermore, histopathological examination shows that high doses of ATZ could influence the seminiferous epithelium. For rats administered average and high doses of ATZ the arrangement of cells was irregular and disordered, and intracellular 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. Thus it can be suggested that following exposure to ATZ, leydig cells would degenerate. Thus disrupts testicular function.

CONCLUSION:

The present study demonstrates that ATZ, irrespective of the dose causes morpho-physiological and testicular dysfunctions with correspondent reduction in sperm quality and circulating gonadotropic hormones.

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