

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: <https://journals.lww.com/apjr/>

doi: 10.4103/apjr.apjr_209_24

Impact of azoxystrobin exposure on sperm morphology, antioxidant defense, reproductive hormones, and testicular histopathology in mice

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ABSTRACT

Objective: To assess the effects of azoxystrobin on sperm quality, hormone levels and testicular structure in Swiss albino mice.

Methods: 48 Swiss albino male mice were divided into 7 groups containing 6 mice in each group except Group VII in which 12 mice were taken. Group I served as the control group and treated with vehicle (distil water for 30 and 60 days). Azoxystrobin at three different doses (125, 250, 500 mg/kg body weight) was orally administered to Group II, III and IV for 30 days and Group V, VI and VII for 60 days. After dose completion, sperm parameters, hormonal profile and testis histology were analysed. Half animals of group VII (500 mg/kg body weight) were left untreated for next 30 days to assess the recovery from reproductive toxicity.

Results: At azoxystrobin 125 and 250 mg/kg, sperm viability, count, and motility remained largely unaffected, but a significant decline was observed at azoxystrobin 500 mg/kg after 30 days. After 60 days, all dose levels led to a significant decrease in sperm parameters. Morphological abnormalities in sperm, such as globular heads, bent or coiled tails, and headless or tailless sperm, were noted to be increased in a dose dependent manner. Antioxidant parameters, serum level of reproductive hormones and steroidogenic enzymes followed a similar trend with non-significant changes at low and medium doses but a marked decline at the high dose after both 30 and 60 days of exposure. Histopathology of testis also revealed degenerative changes in seminiferous tubules and Leydig cell.

Conclusions: Azoxystrobin exposure at high dose (500 mg/kg) affects sperm quality and disrupts testicular function *via* potentially impairing antioxidant defences, deregulating steroidogenesis in Swiss albino male mice.

KEYWORDS: Azoxystrobin; Testis; Male; Mice; Sperm; Testicular structure; Reproductive toxicity

1. Introduction

During recent years, the usage of pesticides has increased

significantly to combat a wide range of pests that threaten crops, public health, and property. The increasing worldwide need for agricultural and farm products rely significantly on chemical pesticides. As a result, they became widespread and can be detected in virtually every aspect of the environment[1,2]. Fungicides constitute more than 40% of the total pesticide sales and are primarily applied to fruits and vegetables to protect them from fungal diseases. In urban environments, fungicides are also being used in human pharmaceuticals, in wood and coating preservatives and in public and private spaces, such as golf courses, lawns, and gardens. As a result of wide exposure of these fungicides, concerns

Key Points

Question: Can exposure to azoxystrobin lead to adverse impact to male reproductive health?

Findings: Exposure of azoxystrobin at high dose (500 mg/kg) significantly reduced sperm count, motility, and viability along with morphological abnormalities (globular heads, bent tails, headless sperm). High-dose exposure led to oxidative stress, potentially impairing antioxidant defense. Testosterone and other reproductive hormones also declined significantly at high doses. Degenerative alterations in seminiferous tubules and Leydig cells were observed, indicating disrupted spermatogenesis and steroidogenesis.

Meaning: Azoxystrobin exposure, especially at higher doses and longer durations, negatively impacts male reproductive health.

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How to cite this article: Rathore H, Pandey G. Impact of azoxystrobin exposure on sperm morphology, antioxidant defense, reproductive hormones, and testicular histopathology in mice. *Asian Pac J Reprod* 2025; 14(6): 271-281.

Article history: Received: 14 November 2024; Revision: 6 April 2025; Accepted: 29 July 2025; Available online: 24 December 2025

have been raised about the health of humans and the environment[3].

Strobilurins is a class of fungicides originating from the mushroom *Strobilurus tenacellus*. It is a class of broad-spectrum fungicides which inhibits electron transfer between cytochrome b and cytochrome c in fungal cells by selectively inhibiting the ubiquinol oxidizing site (Q^o) of mitochondrial respiratory complex III[4]. These fungicides have been widely featured in the portfolios of major pesticide companies and were accounted for nearly one-quarter of the global fungicide market in 2016. Specifically, azoxystrobin, pyraclostrobin, trifloxystrobin, fluoxastrobin, picoxystrobin, and kresoxim-methyl are considered the most widely used strobilurins[5]. Azoxystrobin is considered a top priority for biomonitoring in several nations due to its presence in various sources, including food items and environmental components like soil, water, and air[6]. It has been extensively used in cereals, grapevines, nuts, fruits, and many other vegetables. Despite primarily serving as a fungicide in agriculture, it has also been identified in mold-resistant wallboards, with a concentration of 88.5 µg/g, raising concerns about potential indoor pollution[7].

Based on the available data, azoxystrobin and its formulation were considered as very toxic to aquatic organisms. Parental exposure to azoxystrobin in zebrafish has been linked to increased mortality and developmental abnormalities in their F1 offspring and induced retarded gonadal development, interrupted synthesis of sex hormone[8]. Cao *et al*[9] also recorded decreased egg production, fertilization rates, reduced concentration of 17β-estradiol and vitellogenin along with increase in testosterone concentration in zebrafish exposed to azoxystrobin. Few studies have been conducted to analyse toxicity of azoxystrobin in mammalian models. Exposure of azoxystrobin has been reported to cause disrupted cortical migration in mouse embryos by elevating reactive oxygen species (ROS) levels and triggering mitochondrial deactivation[10] and a reduction in sex hormone production and testicular damage in rats[11]. Moreover, a recent investigation revealed that azoxystrobin efficiently transfers across the placenta from exposed prenatal mice to their offspring[12]. Considering the widespread occurrence of azoxystrobin in the environment and limited research exploring its potential negative effects on the male reproductive system, the present study was designed to assess the potential harm to male reproductive health caused by azoxystrobin and to investigate the underlying mechanisms, with the goal of determining the safety of this fungicide.

2. Methods

2.1. Chemicals

Azoxystrobin (methyl (2E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) was purchased from Rajlaxmi

Pesticides Trading Co., West Bengal, India. All chemicals and reagents utilized in study were of analytical-grade and were procured from Sigma (St Louis, MO, USA).

2.2. Experimental animals

Forty eight Swiss albino male mice (*Mus musculus*) were used as experimental animals. Healthy, adult male mice, 8 weeks old (30–35 g) were kept in an animal house facility of IIS deemed to be University, Jaipur, certified by the Ethical Committee CPCSEA (Registration number: 1689/PO/9/13/CPCSEA). The animals were housed in an air-conditioned room with a temperature of (26±2)°C and a light/dark cycle of 12/12 hours and were provided with maintained on standard diet and water *ad libitum* at the animal house. The guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA) were followed.

2.3. Experimental design

Male mice were divided into seven groups containing 6 animals in each group except Group VII in which 12 animals were taken. Group I was considered as the control group and treated with vehicle (distil water for 30 and 60 days), and Group II, III and IV were treated orally with azoxystrobin at the dose level of 125 mg/kg, 250 mg/kg, 500 mg/kg body weight (b. wt.), respectively, for 30 days and Group V, VI and VII were orally administered with azoxystrobin at the dose level of 125 mg/kg, 250 mg/kg, 500 mg/kg b. wt., respectively, for 60 days. After completion of group VII, half animals were left untreated for recovery for next 30 days. 1/40, 1/20 and 1/10 of LD₅₀ (5000 mg/kg b. wt.) was selected as low, medium and high dose of azoxystrobin.

2.4. Autopsy schedule

After 24 hours of the last dose, all animals were sacrificed under light ether anaesthesia. Testes and cauda epididymis were dissected out. Half tissue of testis was fixed in formalin for histological preparation and remaining tissue was analysed for antioxidants parameters. Cauda epididymal tissue was used for sperm parameters.

2.5. Parameters of the study

2.5.1. Sperm parameters

In each mouse, the cauda epididymis was quickly removed, minced in phosphate buffer saline (PBS; pH 7.2) and incised in order to allow the sperms to exit freely. To assess sperm parameters, approximately 50 µL of sperm concentrate was diluted 20 times with PBS. Sperms were counted using the improved Neubauer haemocytometer. Motile sperms were counted using haemocytometer and percentage of motile sperms was calculated. Sperm viability was

determined using the eosin-nigrosin staining technique described by Prasad *et al*[12]. Sperm smears were prepared from diluted sperm suspension, allowed to dry in air and after following haematoxylin & eosin method slides were examined for sperm morphology.

2.5.2. Steroidogenic enzymes

The 3β hydroxysteroid dehydrogenase (3β -HSD) activity and 17β hydroxysteroid dehydrogenase (17β -HSD) in the testis of mice were evaluated using Talalay's[13].

2.5.3. Hormonal assays

Serum level of testosterone, luteinizing hormone (LH) and follicular stimulating hormone (FSH) was estimated by using commercially available enzyme-linked immunosorbent assay (ELISA) Kit (Cal Biotech).

2.5.4. Antioxidants parameters

Content of ascorbic acid and glutathione (GSH); enzymatic activity of superoxide dismutase (SOD) and catalase along with level of lipid peroxidation in the testis was estimated by the standard methods[14–18].

2.5.5. Histological study

For histological observations, testis was dissected out and rinsed in normal saline and preserved with 10% formalin. After that, tissue was dehydrated in graded series of alcohol, washed in xylene, embedded in paraffin wax, sectioned at 5 μ m in microtome, stained with hematoxylin and eosin (H & E). Tissue sections were examined for histopathological effects under Nikon phase contrast microscope (Nikon Instruments Inc.) at $\times 100$ and $\times 400$ magnification.

2.5.6. Histomorphometry

To measure the diameter of seminiferous tubules, at least 50 round tubules were randomly selected per animal and assessed using a light microscope equipped with an ocular micrometer calibrated with a stage micrometer. Measurements were averaged and expressed as seminiferous tubule diameter. The nuclear diameter of Leydig cells was determined by calculating the average of measurements taken from a minimum of 100 Leydig cell nuclei using the same ocular micrometer. The results were expressed as the mean nuclear diameter of Leydig cells.

2.6. Statistical analysis

Statistical analysis was performed using statistical software package SPSS Version 18.0 for windows. Data were analyzed using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test to compare different treatments. Values were presented as mean $\pm$ standard deviation (mean $\pm$ SD), and differences were judged significant at $P < 0.05$.

2.7. Ethics statement

This study was approved by the Institutional Animal Ethics Committee (IAEC) of IIS deemed to be University, Jaipur (the project proposal No. IAEC/2022/I/4; date: 16 July 2022).

3. Results

3.1. Sperm parameters

At low and medium doses, sperm count, motility and viability showed non-significant changes while mice treated with high dose of azoxystrobin for 30 days showed significant reduction ($P < 0.05$) in sperm count, motility and viability compared to the control group. After 60 days of azoxystrobin treatment, sperm count, motility and viability showed a significant decrease in a dose dependent manner at the level of $P < 0.05$, $P < 0.01$ and $P < 0.001$ for low, medium and high dose, respectively, when compared with the control group.

Intergroup comparison indicated that medium dose group showed non-significant change in sperm parameters after 30 days while a significant ($P < 0.05$) decline in sperm motility and viability after 60 days' exposure as compared to low dose group. High dose group showed a significant decline in sperm count, motility and viability as compared to the low and medium dose groups after 30 days ($P < 0.05$) and 60 days ($P < 0.01$). In recovery group, sperm count, motility and viability were noted to be significantly improved ($P < 0.01$) when compared with high dose group (Table 1).

3.2. Sperm morphology

Morphological variations in sperms of the control and treated groups are shown in Table 2 and Supplementary Figure 1. Various kinds of morphological alterations in sperms head and tail such as coiled/folded tail, amorphous head, headless, tailless, bent tail were observed. In the control group 30 and 60 days, healthy spermatozoa with minor abnormalities were observed. After 30 days of azoxystrobin treatment, no significant change was found in the number of coiled tails, tailless, amorphous head and headless while a significantly dose dependent manner increase ($P < 0.05$; $P < 0.01$; $P < 0.001$) was noted in number of coiled tails, tailless, amorphous head, headless sperm after 60 days of azoxystrobin treatment in comparison to the control group.

Intergroup comparison revealed that number of abnormal sperms was significantly increase ($P < 0.05$) in medium dose group as compared with low dose group only after 60 days of azoxystrobin exposure. Multiple group comparison indicated a significant deviation in the number of sperms with abnormalities after 30 ($P < 0.05$) and 60 ($P < 0.01$) days when high dose group was compared with low and medium dose groups. After 30 days of treatment withdrawal, significant reduction was noted in the number of

Table 1. Effects of different doses of azoxystrobin on sperm parameters in mice at 30 and 60 days of treatment.

Sperm parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
Sperm count, million/mL					
30 days	40.17±0.31	39.67±0.21	38.83±0.18	37.50±0.22 ^{*,#,\$}	-
60 days	45.17±0.14	41.50±0.19 [*]	40.17±0.19 ^{**}	36.17±0.28 ^{***,##,\$\$}	42.17±0.18 ^{&,&}
Sperm motility, %					
30 days	73.50±0.14	73.17±0.13	72.67±0.22	70.17±0.13 ^{*,#,\$}	-
60 days	77.33±0.18	71.33±0.26 [*]	68.83±0.29 ^{**}	63.00±0.24 ^{***,##,\$\$}	71.83±0.23 ^{&,&}
Sperm viability, %					
30 days	63.50±0.12	62.83±0.21	62.00±0.16	59.33±0.18 ^{*,#,\$}	-
60 days	61.83±0.15	57.67±0.13 [*]	56.67±0.06 ^{**}	53.33±0.21 ^{***,##,\$\$}	60.50±0.14 ^{&,&}

Data are expressed as mean±SD. Compared to the control group: ^{*}*P*<0.05, ^{**}*P*<0.01, ^{***}*P*<0.001; Compared to the low dose group: [#]*P*<0.05, ^{##}*P*<0.01; Compared to the medium dose group: ^{\$}*P*<0.05, ^{\$\$}*P*<0.01; Compared to the high dose group: [&]*P*<0.05, ^{&&}*P*<0.01.

Table 2. Effects of different doses of azoxystrobin on sperm morphology in mice at 30 and 60 days of treatment.

Sperm morphology parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
Amorphous head/Globular head, %					
30 days	2.00±0.11	2.33±0.19	2.83±0.27	3.67±0.03 ^{*,#,\$}	-
60 days	3.00±0.15	4.33±0.14 [*]	6.67±0.19 ^{**}	10.67±0.13 ^{***,##,\$\$}	8.00±0.14 ^{*,&,&}
Headless, %					
30 days	1.67±0.26	2.33±0.17	2.59±0.18	4.00±0.18 ^{*,#,\$}	-
60 days	2.00±0.22	6.67±0.18 [*]	9.33±0.30 ^{**}	14.33±0.06 ^{***,##,\$\$}	11.00±0.31 ^{*,&}
Coiled/folded tail, %					
30 days	1.67±0.09	2.00±0.17	2.33±0.18	3.67±0.19 ^{*,#,\$}	-
60 days	2.33±0.14	4.33±0.15 [*]	6.00±0.22 ^{**}	8.67±0.09 ^{***,##,\$\$}	6.33±0.11 ^{*,&}
Tailless, %					
30 days	0.67±0.20	1.07±0.02	2.41±0.16	3.33±0.20 ^{*,#,\$}	-
60 days	1.33±0.20	3.33±0.08 [*]	5.67±0.29 ^{**}	14.33±0.07 ^{***,##,\$\$}	10.67±0.15 ^{*,&}
Bent tail, %					
30 days	1.61±0.27	2.00±0.09	2.67±0.07	3.67±0.05 ^{*,#,\$}	-
60 days	1.67±0.08	3.27±0.23 [*]	5.46±0.29 ^{**}	9.67±0.21 ^{***,##,\$\$}	6.67±0.22 ^{*,&,&}

Data are expressed as mean±SD. Compared to the control group: ^{*}*P*<0.05, ^{**}*P*<0.01, ^{***}*P*<0.001; Compared to the low dose group: [#]*P*<0.05, ^{##}*P*<0.01; Compared to the medium dose group: ^{\$}*P*<0.05, ^{\$\$}*P*<0.01; Compared to the high dose group: [&]*P*<0.05, ^{&&}*P*<0.01.

Table 3. Steroidogenic enzymes parameters in the testis of mice treated with various doses of azoxystrobin for 30 and 60 days.

Enzymes parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
3β-HSD, n moles androstenedione formed/mg protein/min					
30 days	0.56±0.10	0.53±0.19	0.53±0.04	0.51±0.13 ^{*,#,\$}	-
60 days	0.56±0.16	0.50±0.03 [*]	0.47±0.02 ^{**}	0.43±0.12 ^{***,##,\$\$}	0.47±0.02 ^{*,&,&}
17β-HSD, n moles androstenedione formed/mg protein/min					
30 days	0.47±0.15	0.45±0.12	0.44±0.07	0.43±0.09 ^{*,#,\$}	-
60 days	0.50±0.24	0.43±0.06 [*]	0.40±0.15 ^{**}	0.38±0.14 ^{***,##,\$\$}	0.44±0.05 ^{*,&,&}

Data are expressed as mean±SD. Compared to the control group: ^{*}*P*<0.05, ^{**}*P*<0.01, ^{***}*P*<0.001; Compared to the low dose group: [#]*P*<0.05, ^{##}*P*<0.01; Compared to the medium dose group: ^{\$}*P*<0.05, ^{\$\$}*P*<0.01; Compared to the high dose group: [&]*P*<0.05, ^{&&}*P*<0.01. HSD: hydroxysteroid dehydrogenase.

amorphous head (*P*<0.01), headless (*P*<0.05), coiled tail (*P*<0.05), tailless (*P*<0.05), and bent tail (*P*<0.01) (Table 2).

3.3. Steroidogenic enzymes

Activity of 3β-HSD and 17β-HSD in testis of male mice declined significantly only in the high dose treatment group after 30 days treatment while both activity of 3β-HSD and 17β-HSD showed a dose dependent reduction in testis of male mice after 60 days exposure when compared with the control mice. Within a group

comparison result showed that 3β-HSD and 17β-HSD activity was significantly (*P*<0.05; *P*<0.01) depleted in the high dose group (500 mg/kg b.wt./day) when compared with both low (125 mg/kg b.wt./day) and medium (250 mg/kg b.wt./day) dose groups after 30- and 60-days exposure, respectively. The activity of both enzymes revealed a significant decline (*P*<0.05) in medium dose group in comparison to low dose group after 60 days exposure only. In the recovery group mice, activity of 3β-HSD and 17 β-HSD was noted to be significantly (*P*<0.01) higher than high dose group, but still significant (*P*<0.05) less than the control group (Table 3).

Table 4. Hormone parameters in the serum of mice treated with various doses of azoxystrobin for 30 and 60 days.

Hormonal parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
Testosterone, ng/mL					
30 days	5.41±0.15	5.20±0.13	4.98±0.05	4.90±0.19 ^{*,#}	-
60 days	5.26±0.08	4.60±0.15 [*]	4.40±0.04 ^{*,#}	3.68±0.21 ^{***,#,S}	4.32±0.26 ^{*,&&}
Luteinizing hormone (LH), mIU/mL					
30 days	2.60±0.31	2.40±0.23	2.20±0.24	1.95±0.17 ^{*,#S}	-
60 days	2.86±0.12	2.21±0.20 [*]	2.00±0.14 ^{***}	1.08±0.19 ^{***,##,SS}	1.74±0.06 ^{*,&&}
Follicular stimulating hormone (FSH), mIU/mL					
30 days	3.60±0.32	3.40±0.08	3.08±0.17	2.66±0.22 ^{*,#S}	-
60 days	4.00±0.09	3.08±0.19 [*]	2.68±0.26 ^{***}	1.93±0.09 ^{***,##,SS}	2.84±0.15 ^{*,&&}

Data are expressed as mean±SD. Compared to the control group: ^{*} $P<0.05$, ^{**} $P<0.01$, ^{***} $P<0.001$; Compared to the low dose group: [#] $P<0.05$, ^{##} $P<0.01$; Compared to the medium dose group: ^S $P<0.05$, ^{SS} $P<0.01$; Compared to the high dose group: ^{&&} $P<0.01$.

Table 5. Antioxidant defence system markers in the testis of mice treated with various doses of azoxystrobin for 30 and 60 days.

Antioxidant parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
SOD, unit/mg protein					
30 days	17.50±0.50	16.67±1.05	15.00±0.23	14.17±1.07 ^{*,#S}	-
60 days	14.33±0.88	10.50±0.92 [*]	8.50±0.81 ^{***}	7.83±0.48 ^{***,##,SS}	13.17±2.04 ^{&&}
Catalase, p moles H ₂ O ₂ consumed/mg protein/min					
30 days	19.50±1.62	18.67±2.81	17.83±1.66	16.00±1.70 ^{*,#S}	-
60 days	17.17±1.29	12.00±2.65 [*]	9.83±2.42 ^{***}	8.17±1.93 ^{***,##,SS}	16.17±0.89 ^{&&}
GSH, n mol/g tissue					
30 days	4.51±1.19	4.50±0.55	4.49±0.29	4.47±1.78 ^{*,#S}	-
60 days	4.44±0.26	4.34±0.74 [*]	4.32±0.65 ^{***}	4.21±1.44 ^{***,##,S}	4.39±0.29 ^{*,&&}
Ascorbic acid, mg/g tissue					
30 days	1.61±0.26	1.59±0.03	1.56±0.13	1.52±0.14 ^{*,#S}	-
60 days	1.60±0.29	1.50±0.04 [*]	1.45±0.71 ^{***}	1.34±0.24 ^{***,##,S}	1.29±0.22 ^{*,&&}
LPO, n mol MDA mg/tissue					
30 days	10.83±1.64	11.67±0.79	12.50±1.48	13.83±1.57 ^{*,#S}	-
60 days	11.17±0.44	14.50±1.08 [*]	16.33±0.08 ^{***}	19.17±1.39 ^{***,##,SS}	10.50±1.73 ^{&&}

Data are expressed as mean±SD. Compared to the control group: ^{*} $P<0.05$, ^{**} $P<0.01$, ^{***} $P<0.001$; Compared to the low dose group: [#] $P<0.05$, ^{##} $P<0.01$; Compared to the medium dose group: ^S $P<0.05$, ^{SS} $P<0.01$; Compared to the high dose group: ^{&&} $P<0.01$, ^{&&&} $P<0.001$. SOD: superoxide dismutase; GSH: glutathione; LPO: lipid peroxidation.

3.4. Male reproductive hormones

After 30 days of azoxystrobin exposure, serum level of testosterone, FSH and LH in adult male albino mice showed significant reduction ($P<0.05$) only at high dose level *i.e.* 500 mg/kg b.wt in comparison to the control group. The testosterone, FSH and LH level declined in a dose dependent manner in male mice treated with divergent doses of azoxystrobin for 60 days when compared with the control group. Multiple comparison tests among different azoxystrobin treatment groups showed that after 30 days exposure, high dose group indicated significant reduction ($P<0.05$) in hormonal level in comparison to low dose only. After 60 days' administration of azoxystrobin, a significant decline was noted in testosterone ($P<0.05$) FSH and LH level ($P<0.01$) in high dose group when compared with low and medium dose groups. Medium dose group showed significant reduction ($P<0.05$) in LH level only after 60 days exposure when compared with low dose group. Serum testosterone, FSH and LH level showed significant improvement ($P<0.01$) in recovery group when compared with high dose group while it was significantly lower ($P<0.01$) than the control group (Table 4).

3.5. Antioxidant parameters

Mice treated with azoxystrobin for 30 days showed non-significant change in antioxidants level at low and medium dose level while significant decline was observed at high dose ($P<0.05$). After 60 days exposure of azoxystrobin, activity of SOD, catalase as well as concentration of GSH and ascorbic acid in testis was decreased in a dose dependent manner when compared with the control group. The multiple comparison tests among the azoxystrobin treated groups revealed a significant ($P<0.05$; $P<0.01$) decline in content of GSH and ascorbic acid and reduction in activity of SOD and catalase in high dose group when compared to the low dose group after exposure of 30 and 60 days, respectively, while medium dose group showed a significant ($P<0.05$) decline in the all-antioxidant parameters in testis of mice only after exposure at 60 days when compared to the low dose group. In the recovery group, antioxidant parameters found to be increased significantly when compared to the 500 mg/kg b.wt dose level (Table 5).

3.6. Histopathology of testes

The histological picture of the testis in the control mice displayed normal internal structure, exhibiting uninterrupted spermatogenesis (Figure 1 A, B). After 30 days of azoxystrobin treatment with low dose, the histoarchitecture of testis showed slight degenerative changes in germinal epithelium (Figure 1 C, D). Medium dose-treated mice showed mild degenerative changes and slight decline in number of spermatozoa in lumen (Figure 1 E, F). The testicular histoarchitecture of the mice treated with high dose of azoxystrobin showed disorganized seminiferous epithelium and reduced germ cell populations, increased inter-tubular spaces and scanty spermatozoa in seminiferous tubules. The Leydig cells also showed degenerative and atrophic changes with reduced nuclear diameter (Figure 1 G, H).

After 60 days of treatment the histopathological photomicrograph of the testis showed dose and duration dependent degenerative and atrophic changes in the seminiferous tubules and Leydig cells. Testicular section of the control mice exhibited normal histoarchitecture (Figure 2 A, B). Histoarchitecture of testis in low dose group showed degenerative and atrophic changes with reduced number of germ cell populations and spermatozoa in the lumen of seminiferous tubules (Figure 2 C, D). In medium dose group, the histological picture of the testis showed noticeable degenerative and atrophic alteration in the seminiferous tubules and Leydig cells and disorganization and sloughing of germ cells, diminished germ cell population and tubular diameter along with increased inter-tubular space were noted at higher magnification (Figure 2 E, F). The degree of degeneration in the testis was further increased in high dose group as depicted by severe disintegration and diminution of spermatogenic cells, sloughing of germ cells, shrinkage of seminiferous tubules as well as presence of sperm debris, almost absence of spermatozoa in the lumen and

degenerative and atrophic changes in Leydig cells (Figure 2 G, H).

After 60 days of the withdrawal of treatment in the high dosage group, the testicular histoarchitecture represented a significant improvement depicting near normal appearance of seminiferous tubules, greater quantity of spermatozoa in the lumen and almost normalized Leydig cell morphology. However, mild degenerative changes in later stages of spermatogenic cells were still persistent (Figure 2 I, J).

3.7. Histomorphometry

After 30 days of azoxystrobin exposure, the low and medium dose groups did not show any significant change in the seminiferous tubule diameter and nuclear diameter of Leydig cells while a significant reduction in seminiferous tubule diameter and nuclear diameter of Leydig cells in high dose group were shown as compared to the control group ($P<0.05$). After 60 days of azoxystrobin exposure, seminiferous tubular and Leydig cell nuclear diameter showed a significant ($P<0.05$, $P<0.01$, and $P<0.001$) dose-dependent decrease as compared to the control group. Seminiferous tubular and Leydig cell nuclear diameter showed a significant ($P<0.05$; $P<0.01$) reduction in high dose group when compared to the low and medium dose groups after exposure of 30 and 60 days, respectively, during inter group comparison while at medium dose level, both of these showed a significant reduction ($P<0.05$) only after exposure of 60 days, when compared to the low dose group. Withdrawal of azoxystrobin treatment (500 mg/kg b.wt.; 60 days) for 30 days resulted in significant improvement in seminiferous tubule diameter ($P<0.05$) and Leydig cell nuclear diameter ($P<0.01$) as compared to respective treatment group. However, the recovery could not reach to the control value (Table 6).

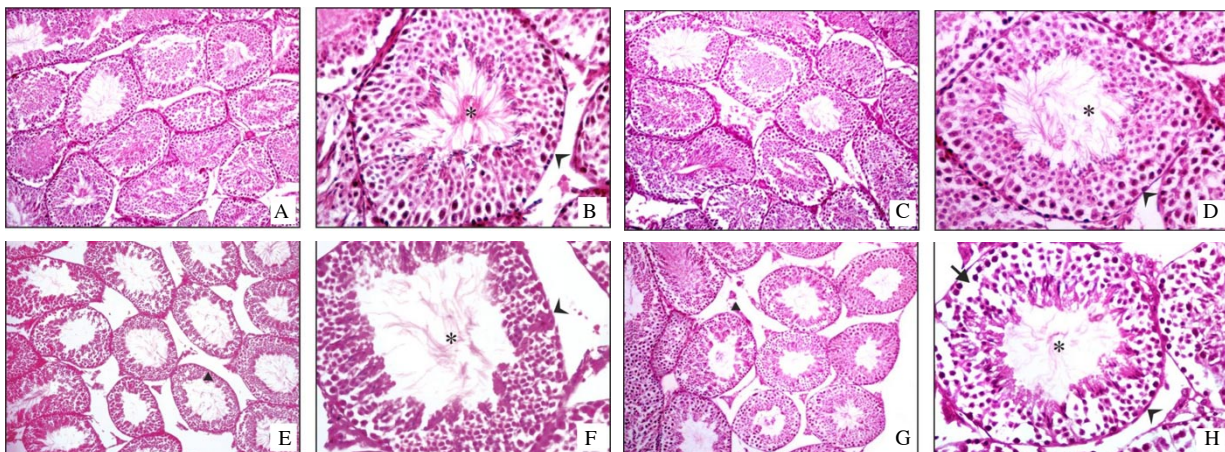


Figure 1. Photomicrographs show histopathology of testes of the control mice and the mice treated with azoxystrobin at three different doses (125, 250, 500 mg/kg body weight) for 30 days. A: Photomicrograph of the testicular section of the control mice exhibiting normal histoarchitecture (H & E, $\times 100$); B: Photomicrograph of the same at higher magnification (H & E, $\times 400$); C: Photomicrograph of the cross section of testis in azoxystrobin (125 mg/kg b.wt.) treated mice showing slight degenerative changes in seminiferous tubules (H & E, $\times 100$); D: Photomicrograph of the same at higher magnification (H & E, $\times 400$); E: Photomicrograph of the cross section of testis in azoxystrobin (250 mg/kg b.wt.) treated mice showing mild degenerative changes and slight decline in number of spermatozoa in lumen (H & E, $\times 100$); F: Photomicrograph of the same at higher magnification (H & E, $\times 400$); G: Photomicrograph of the cross section of testis of azoxystrobin (500 mg/kg b.wt.) treated mice showing marked degenerative and atrophic changes with reduced number of spermatozoa in the lumen (H & E, $\times 100$); H: Photomicrograph of the same at higher magnification (H & E, $\times 400$). * Spermatozoa; ▲ Seminiferous tubule; ▲ Degeneration; ▲ Interstitial space.

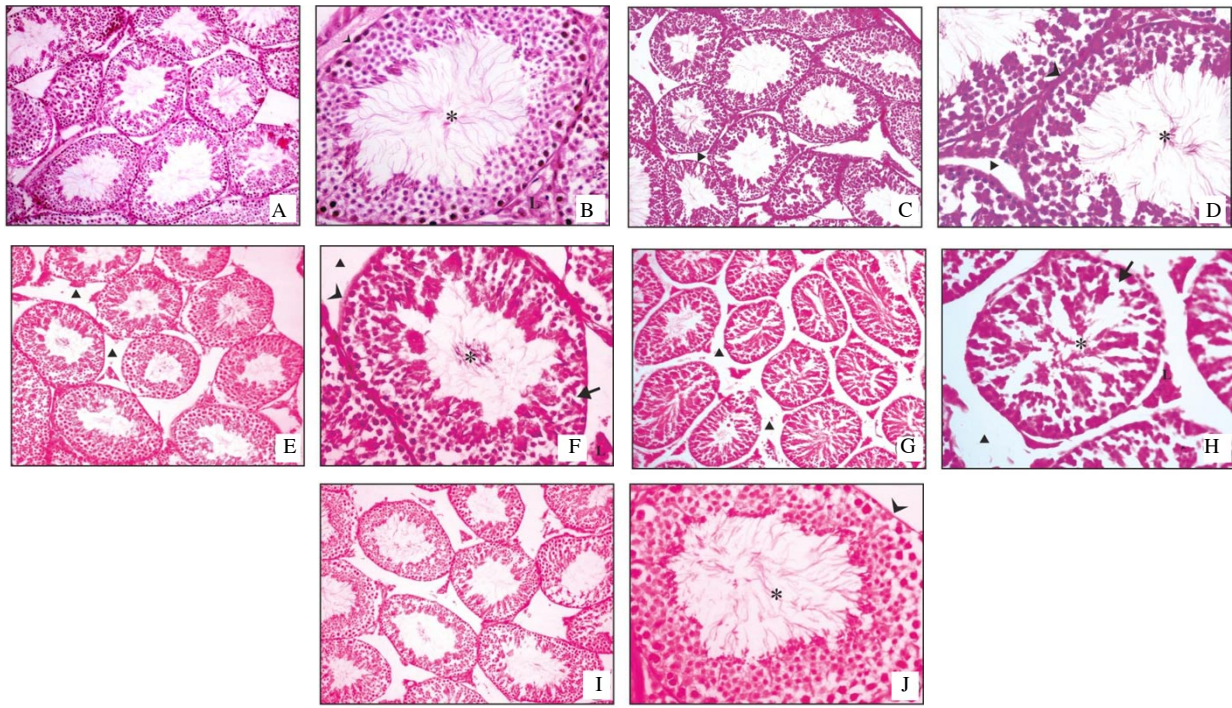


Figure 2. Photomicrographs show histopathology of testes of the control mice and the mice treated with azoxystrobin at three different doses (125, 250, 500 mg/kg body weight) for 60 days. A: Photomicrograph of testicular section of the control mice exhibiting normal histoarchitecture (H & E, $\times 100$); B: Photomicrograph of the same at higher magnification (H & E, $\times 400$); C: Photomicrograph of the cross section of testis in azoxystrobin (125 mg/kg b.wt.) treated mice showing degenerative and atrophic changes in seminiferous epithelium along with impairment of spermatogenesis (H & E, $\times 100$); D: Photomicrograph of the same at higher magnification (H & E, $\times 400$); E: Photomicrograph of the cross section of testis in azoxystrobin (250 mg/kg b.wt.) treated mice showing marked degenerative and atrophic changes in the seminiferous tubules and Leydig cells (H & E, $\times 100$); F: Photomicrograph of the same at higher magnification (H & E, $\times 400$); G: Photomicrograph of the cross section of testis in azoxystrobin (500 mg/kg b.wt.) treated mice displaying severely damaged seminiferous tubules and degenerating Leydig cells (H & E, $\times 100$); H: Photomicrograph of the same at higher magnification (H & E, $\times 400$); I: Photomicrograph of cross section of testis in mice after 30 days of withdrawal of azoxystrobin treatment (500 mg/kg b.wt./day) withdrawal showing apparently normal histoarchitecture of seminiferous tubules (H & E, $\times 100$); J: Photomicrograph of the same at higher magnification (H & E, $\times 400$). * Spermatozoa; ▲ Seminiferous tubule; L Leydig cell; ▲ Interstitial space; ▲ Degeneration.

Table 6. Histomorphometric indices of seminiferous tubule and Leydig cell nuclear diameter of mice treated with various doses of azoxystrobin for 30 and 60 days.

Histomorphometric parameters	Control	Low dose (125 mg/kg b.wt.)	Medium dose (250 mg/kg b.wt.)	High dose (500 mg/kg b.wt.)	Recovery
Seminiferous tubule diameter, μm					
30 days	142.00 $\pm$ 2.75	141.00 $\pm$ 3.17	139.16 $\pm$ 1.83	136.77 $\pm$ 0.82 ^{*,#,\$}	-
60 days	145.00 $\pm$ 3.48	140.13 $\pm$ 0.16 [*]	137.35 $\pm$ 2.58 ^{*,#}	121.24 $\pm$ 2.47 ^{***,##,\$\$}	127.11 $\pm$ 2.57 ^{***,&}
Leydig cell nuclear diameter, μm					
30 days	5.16 $\pm$ 0.32	5.08 $\pm$ 1.17	4.91 $\pm$ 0.38	4.86 $\pm$ 1.14 ^{*,#,\$}	-
60 days	5.32 $\pm$ 1.82	4.93 $\pm$ 1.15 [*]	4.82 $\pm$ 0.87 ^{*,#}	4.63 $\pm$ 1.34 ^{***,##,\$}	4.74 $\pm$ 0.42 ^{***,&&}

Data are expressed as mean $\pm$ SD. Compared to the control group: ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$; Compared to the low dose group: [#] $P < 0.05$, ^{##} $P < 0.01$; Compared to the medium dose group: ^{\$} $P < 0.05$, ^{\$\$} $P < 0.01$; Compared to the high dose group: [&] $P < 0.05$, ^{&&} $P < 0.01$.

4. Discussion

Semen parameters such as sperm count, motility and viability are analysed to assess male fertility. An abnormal sperm count, poor sperm motility and a high number of dead or non-moving sperm lower the chance of natural conception[19]. In the current study, sperm count, motility and viability were noted to be decreased with increasing dose and duration of azoxystrobin exposure. During inter group comparison, the high dose group treated with azoxystrobin

revealed significant decline in sperm parameters in comparison to the medium and low dose groups, indicating that exposure to azoxystrobin at high dose level for long duration might disturb sperm parameters. Deterioration in sperm parameters might be due to structural destruction of testes and elevation of oxidative stress level within testes. Azoxystrobin might also disturb the sperm optimum functioning by interfering with production of sex hormones. Therefore, looking at the results, it can be inferred that azoxystrobin not only interferes with the production of male sex

hormones (testosterone, FSH and LH) but also deregulates the various components that aid in sperm optimum functioning as well as its survival. Similar findings were also reported by El-Hak *et al*[10] who revealed a decrease in the sperm count and motility in male rats exposed to azoxystrobin and acetamiprid for 30 and 60 days by enhancing oxidative stress in testis which disturb the lipid bilayer in the cell membrane or *via* decreasing testosterone biosynthesis. Sperm morphology is considered as a reliable marker which reflects the impact of toxicants on testicular health. Various abnormalities in sperm head (amorphous head, globular head, headless) and tail (tailless, bent and coiled tail) were observed in mice treated with azoxystrobin at dose level of 500 mg/kg b.wt. Sperm structural deformities might be correlated with enhanced level of free radicals, higher DNA fragmentation index and chromosome aneuploidy[20].

Testes mainly secrete two steroid androgens-testosterone and dihydrotestosterone. Apart from them, a number of weaker androgens like dehydroepiandrosterone (DHEA) and androstenedione are also secreted which are converted to metabolically active testosterone by the action of steroidogenic enzymes. The process of steroidogenesis mainly occurs in Leydig cells in testis under the influence of LH and is catalysed by steroidogenic enzymes. Testes harbor mainly two types of steroidogenic enzymes - cytochrome P450 heme containing proteins and hydroxysteroid dehydrogenases (3β -HSD and 17β -HSD)[21]. The very first step of testosterone biosynthesis is the conversion of cholesterol to pregnenolone catalysed by cytochrome P450_{scc} (cholesterol side-chain cleavage enzyme) in inner mitochondrial membrane. Then, pregnenolone/dehydroxypregnenolone and DHEA are converted to hydroxyprogesterone and androstenedione, respectively. 17β -HSD catalyzes the last step of testosterone biosynthesis by converting androstenedione to testosterone[22]. Activity of 3β -HSD and 17β -HSD in testis of male mice treated with different doses of azoxystrobin declined significantly after 60 days treatment in a dose dependent manner. Multiple group comparison results showed that the azoxystrobin treatment at high dose level cause significant decline in the activity of 3β -HSD and 17β -HSD enzymes as compared to low and medium dose groups. This decline in 3β -HSD and 17β -HSD content in response to azoxystrobin manifests azoxystrobin-mediated reproductive toxicity in mice which is evident from dwindling testosterone levels in a dose dependent manner. Both 3β -HSD and 17β -HSD are the key enzymes in testosterone biosynthesis from cholesterol; however, in the current study we also found increment in the level of total cholesterol in testis of azoxystrobin treated mice. Both of these factors, cholesterol increment and β -HSD enzyme inactivity, act in conjunction with each other leading to reduced testosterone biosynthesis and compromised reproductive ability in azoxystrobin treated mice. Deficiency/inactivity of these enzymes have been linked with decreased testosterone production, which may affect the process of

spermatogenesis and sperm maturation. Improved activity of 3β -HSD and 17β -HSD enzymes in recovery group after withdrawal of treatment may be attributed to re-establishment of treatment-induced stress on cholesterol level and enzyme activity[22].

Hypothalamic-pituitary-testicular axis plays a pivotal role in mediating the complex interplay of events that regulate reproductive activity and release of sex hormones from gonads/testis[23]. Results in our study show inhibitory effect of fungicide azoxystrobin on testosterone level of serum in a dose dependent manner after 60 days treatment in adult male albino mice which may be attributed to perturbation of hypothalamic-pituitary-testicular axis[23]; direct effect on Leydig cells[24]; increased ROS level[25]; detrimental effect on cholesterol transport into the mitochondria of Leydig cells[26]. Many previous studies have also shown similar results where researchers demonstrated decline in testosterone levels in response to treatment with endocrine disrupting chemicals (EDCs)[27,28].

Azoxystrobin mediated decline in LH and FSH activity might be due to perturbation of hypothalamic-pituitary-testicular axis. This axis is responsible for maintaining reproductive hormonal balance crucial for the process of spermatogenesis and sperm maturation. Induction of oxidative stress may trigger disturbance in hypothalamic-pituitary-testicular axis. This process leads to impaired secretion of FSH and LH by the pituitary gland due to miscommunication between hypothalamus and pituitary gland, as a consequence of which, spermatogenesis and overall reproductive health is profoundly affected. A number of previous studies have demonstrated decline in LH and FSH levels in response to treatment with EDCs[29,30]. Inhibitory effect of azoxystrobin on testosterone, LH and FSH was somewhat ameliorated in the recovery group of mice which can be correlated with reduced oxidative stress.

SOD constitutes the first line of defence against ROS in the cell by catalysing the conversion of superoxide radical anions to molecular oxygen and hydrogen peroxide (H_2O_2)[31]. A significant decrease in the level of SOD activity in the testis might be due to its excess utilization in catalysing dismutation of superoxide anion radicals or reduced synthesis of the enzyme proteins or due to excessive generation of free radicals. These observations are also strengthened by other reports which have also shown a significant decline in the SOD activity in azoxystrobin treated rats[10]. Catalase is a heme-containing peroxisomal enzyme that selectively is vital for protecting cells, including spermatozoa, from the harmful effects of H_2O_2 and other ROS. Decrease in the level of catalase might be correlated with intensive increase in superoxide radical and H_2O_2 and thus lead to damage in the testis. Reduction in the catalase activity affects the detoxification of H_2O_2 which in turn leads to the damage of the sperm viability and motility[31]. Testis contain huge amount of GSH which play an important role in nucleus condensation and spindle microtubule polymerization[32]. The reduced levels of GSH indicate insufficiency of antioxidant system which may be caused

due to increased utilization of GSH in trapping excessive generated free radicals as a consequence of toxic effect of azoxystrobin in testis. Ascorbic acid is an essential cofactor for many enzymes involved in various metabolic pathways. It is also necessary for maintaining the physiological integrity of the androgen dependent target reproductive organs[33]. The depletion in ascorbic acid noticed in this study might be due to increased utilization of it to scavenge excessively generated free radicals or its reduced biosynthesis. Ziada *et al*[34] noticed similar changes in antioxidant parameters in liver and kidney along with increased liver and renal function bioindicator (ALT, AST, ALP, urea and creatinine) in male albino rats exposed to azoxystrobin fungicide. The deficiency of ascorbic acid might lead to a state of oxidative stress that disrupts both steroidogenesis and spermatogenesis in the testis[35]. In recovery group, after 30 days of treatment withdrawal, reduced SOD, catalase and ascorbic acid levels were restored in cells which might be due to suppressed free radical formation. Malondialdehyde (MDA), one of the end products of lipid peroxidation in the cell acts as an important indicator for membrane lipid peroxidation. MDA levels are generally measured by thiobarbituric acid-reactive substances (TBARs) assay[36]. Enhanced lipid peroxidation might be due to reduced level of antioxidants as observed in the study. Ziada *et al*[34] reported similar decline in GSH and elevation in MDA level in liver and kidney of male rats administered with azoxystrobin (5 mL/kg b.wt.) up to 28 days.

Histopathology of testis is considered to be the most appropriate parameter for the assessment of harmful effects of any toxicant. The impairment of male fertility due to the exposure of various pesticides and fungicides is a consequence of disrupted activity of androgenic hormones. The histopathological results shown in this study are reflective of the testicular damage in the male mice testis induced by exposure of azoxystrobin for long duration *i.e.* 60 days.

Changes in testicular architecture by azoxystrobin exposure may be a direct consequence of the decline in level of the male key hormone, testosterone, which is responsible for maintaining the normal architecture as well as functioning of the testis. The decline in amount of testosterone may be attributed to azoxystrobin-induced decline in level of steroidogenic enzymes (3β -HSD and 17β -HSD) involved in biosynthesis of testosterone from cholesterol. Another key factor responsible for testicular degeneration and atrophy is detrimental effect of azoxystrobin exposure on the mitochondrial membrane, leading to huge oxidative burst coupled with release of ROS and consequent deterioration of testicular architecture by free radicals. Similar results showing degradative changes and testicular atrophy coupled with deterioration of seminiferous tubules and Leydig cells in mice in response to treatment with fungicides and insecticides owing to increased ROS production have been shown in previous studies[37,38]. Furthermore, impairment of hypothalamic-pituitary-testicular axis by azoxystrobin may be responsible for decreased production of FSH and LH, both of which affect the

functioning as well as morphology of Leydig cells and Sertoli cells, thereby leading to overall degradation of testicular histoarchitecture. Similar results showing degradative changes and testicular atrophy coupled with deterioration of seminiferous tubules and Leydig cells in mice in response to treatment with fungicides and insecticides owing to impairment of hypothalamic-pituitary-testicular axis have been shown in previous study[39]. Improved histopathology of testis in recovery group mice might be due to decline in lipid peroxidation, reduction in oxidative stress in the testis as well as increased levels of circulatory testosterone.

Morphometric analysis acts as an important parameter which provides information about toxicity at specific target cell. The seminiferous tubular diameter is used as a crucial parameter for assessment of spermatogenic activity in testis. Changes observed in the seminiferous tubular diameter in azoxystrobin-treated mice might be due to reduction of secretion of seminiferous tubular fluid, disrupted hypothalamic pituitary gonadal (HPG) axis or reduced level of testosterone or induced oxidative stress. There is strong evidence that shows a positive relationship between diameter of seminiferous tubules and spermatogenesis. Leydig cells act as the principal source of androgens in testis which regulate secondary sexual characters, spermatogenesis and functioning of accessory reproductive glands in male[40]. Reduced Leydig cell nuclear diameter might also be correlated with excessive production of ROS and dysfunction of seminiferous tubules which might be responsible for reduced level of testosterone[41]. However, the diameter of seminiferous tubules and Leydig cell nucleus was also reversed considerably after 30 days of recovery period in high dose-treated mice for 60 days which were in complete concordance with the histopathological results.

The study addresses a critical issue related to the potential reproductive toxicity of a widely used agricultural fungicide, which has implications for both environmental and human health. Present study primarily focused on biochemical, histological and sperm alterations but underlying molecular pathways including gene expression analysis, apoptotic markers and DNA fragmentation and mating success were not evaluated. Therefore, further research is required to validate the results and assess their relevance to human health.

There are some study limitations. In present study, effects of azoxystrobin were evaluated on male reproductive system after exposure of three different doses for 30 and 60 days in mice. The study focused on sperm parameters and testicular histology through assessing oxidative stress, hormone levels, and steroidogenic enzymes parameters, but we did not study molecular mechanisms—such as gene expression profiling, mitochondrial dysfunction, or apoptosis pathways—and mating behavior, fertility rate and litter size. Future studies should be conducted to provide a more comprehensive understanding and confirmation of azoxystrobin's reproductive toxicity.

In conclusion, the results of this study suggest that prolonged exposure to high doses of azoxystrobin, a widely used agricultural fungicide, may negatively impact male fertility. This is evidenced by increased sperm morphological abnormalities, reduced level of male reproductive hormonal level along with altered histopathology of the testes. Therefore, it is crucial to raise awareness among farmers regarding the potential side effects of such fungicides. Additionally, further research is needed to fully establish and confirm its toxic effects.

Conflict of interest statement

Authors declare that they have no conflicts of interest.

Acknowledgements

Authors are highly acknowledged to WISE-KIRAN division of Department of Science and Technology, New Delhi, India for providing infrastructural financial support to IIS (deemed to be university), Jaipur, India (File No. DST/CURIE-02/2023/IISU) under CURIE program.

Funding

The research was conducted without any financial support from external funding agency.

Authors' contributions

Rathore Harkesh contributed to writing original draft, methodology, and formal analysis; Pandey Geeta contributed to writing original draft, review, editing, supervision, and conceptualization.

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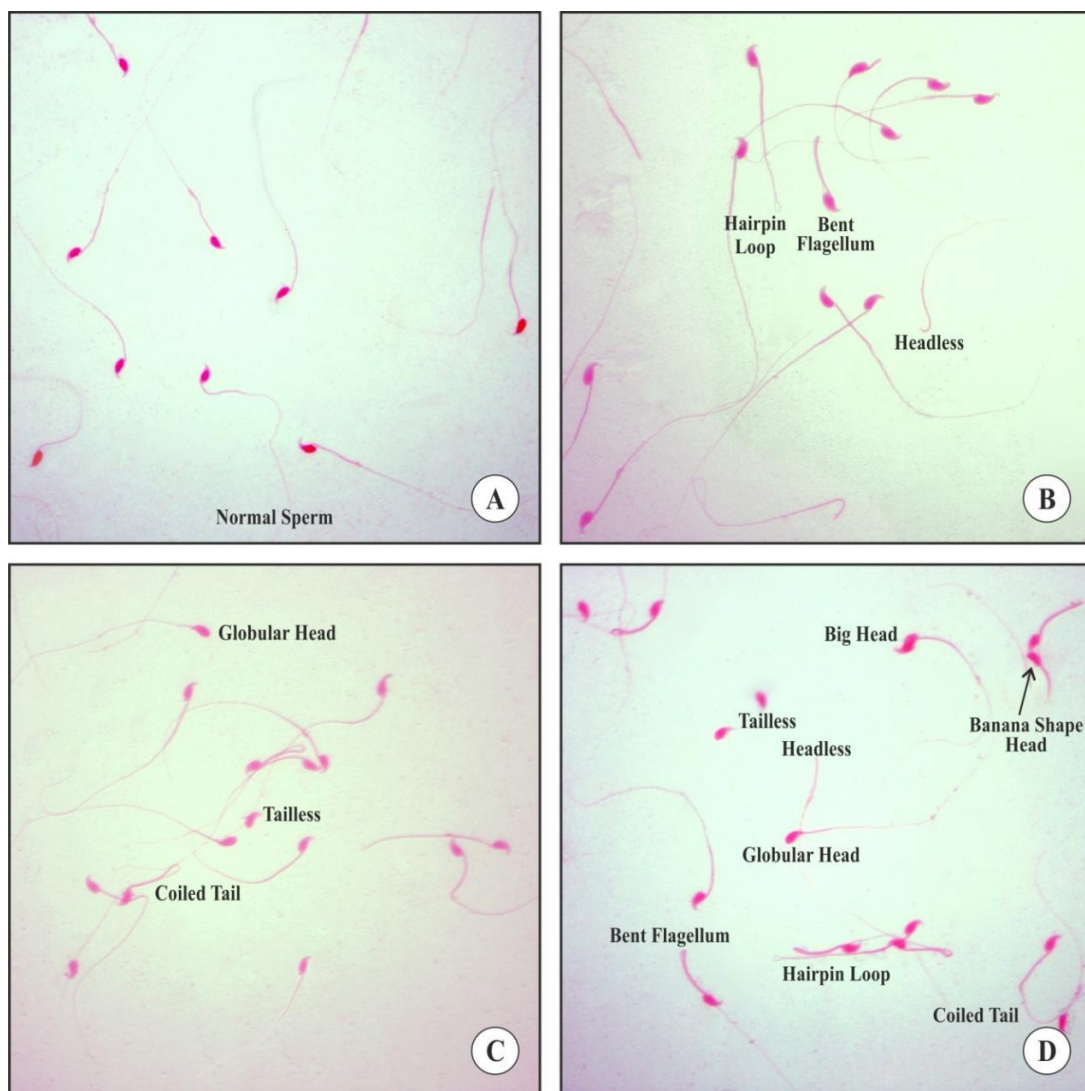
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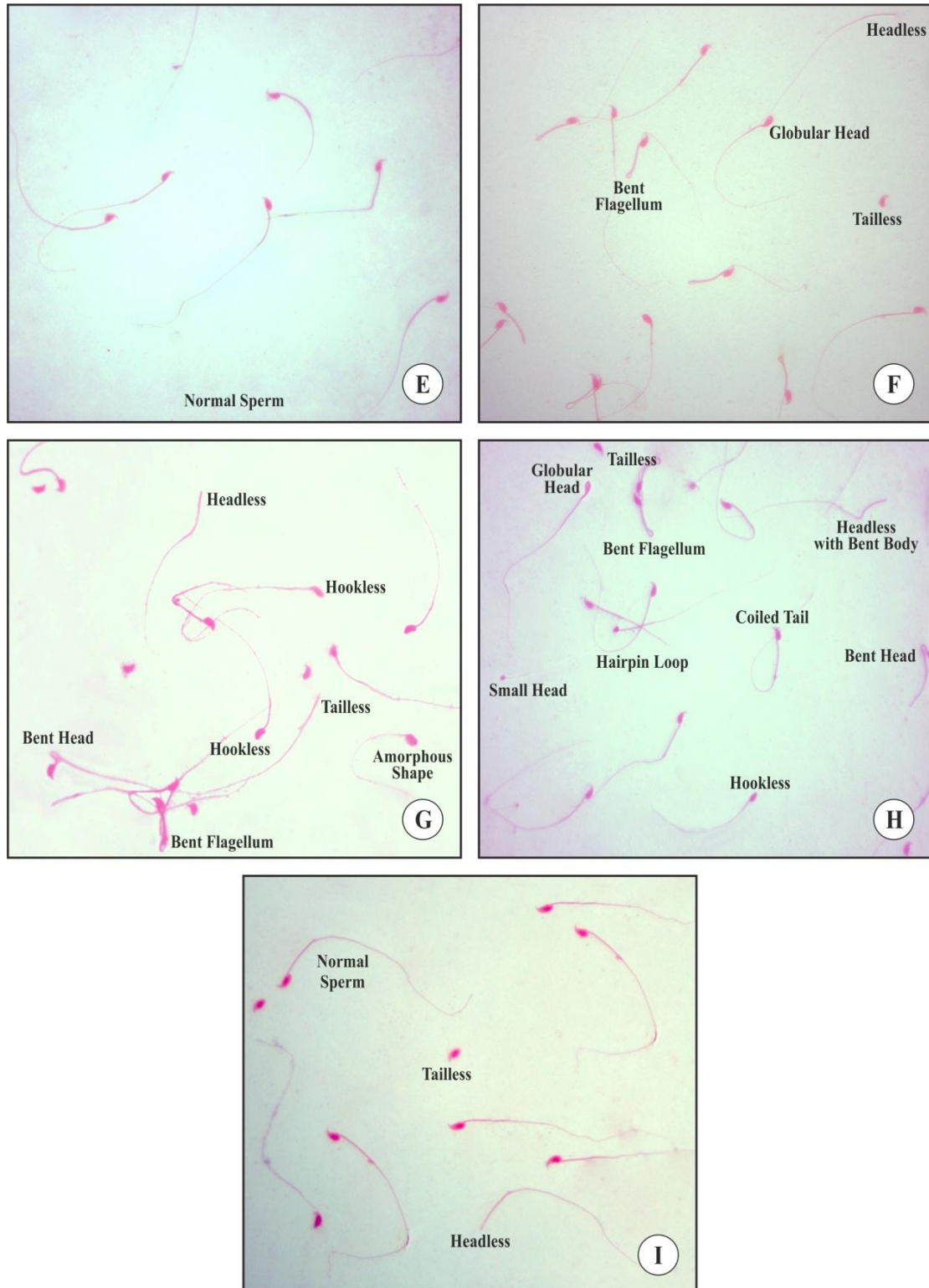
Impact of azoxystrobin exposure on sperm morphology, antioxidant defense, reproductive hormones, and testicular histopathology in mice

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Supplementary Materials





Supplementary Figure 1. Sperm morphology of the control mice and azoxystrobin-treated mice at three different doses (125, 250, 500 mg/kg body weight) for 30 days (A-D) and 60 days (E-I). A) represents the control group mice with normal sperm; B, C) represent slight deformities in sperm morphology in azoxystrobin-treated mice

with low and medium doses (125, 250 mg/kg), and D) represents abnormalities in sperm in azoxystrobin-treated mice with high dose (500 mg/kg) for 30 days. E) represents normal and healthy sperms in the control group mice; F, G) show some sperm deformities in azoxystrobin-treated mice with low and medium doses (125, 250 mg/kg), and H) represents various deformities such as globular head, headless, hookless, small head, bent head, tailless, bent flagellum, coiled tail, *etc.* in sperm of azoxystrobin-treated mice with high dose (500 mg/kg) for 60 days. I) represents normal sperm with minor deformities in sperm in the recovery group.