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ORIGINAL ARTICLE

Effects of Azoxystrobin on Body Weight, Organ Weight and Biochemical Parameters in Testis of Male Mice

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ABSTRACT
Pesticides are being used to protect crops from various pests. These help in improving crop yield but have deleterious effects on environment, health of aquatic and other animals. Azoxystrobin is commonly used fungicide in agriculture. In the present study, effects of azoxystrobin were observed on adult male mice. Effects of azoxystrobin exposure 30 and 60 days at three different doses (125, 250 and 500 mg/kg body weight) were observed on body weight and reproductive organs along with biochemical parameters in testis. Results revealed that significant change was observed only at high dose level after 30 days of treatment while when treatment was given for 60 days, dose dependent significant reduction was observed in body and organ weight. Total protein content and activity of alkaline phosphatase, acid phosphatase and succinate dehydrogenase revealed significant reduction while Cholesterol content was noted to be significantly increased in the testis of all the treated groups for 60 days. Based on results, it can be concluded Azoxystrobin exposure for long term might induce adverse impact on male reproductive system therefore more studies should be planned to explore the underlying mechanism.
Keywords: *Azoxystrobin; Mice; Testis; Biochemical; Male*

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INTRODUCTION

Insects, weeds, rodents, fungi, and other pests can be prevented, controlled, or eliminated with the help of pesticides, which are toxic chemical substances or mixtures of substances or biological agents deliberately introduced into the environment for this purpose. Pesticides entice pests with food or shelter before killing or otherwise reducing their population [1]. Different pesticide classes are designed to kill certain pests and have varying degrees of toxicity for nontarget creatures. Numerous "minor" classes exist, such as acaricides (mites) or molluscides (snails, other mollusks), in addition to the four primary classes (and their target pests) of insecticides (insects), herbicides (weeds), fungicides (fungi, molds), and rodenticides (rodents). Several subclasses exist within each class, each having its own unique chemical and toxicological properties [2]. Fungicides are used to protect tubers, fruits, and vegetables during storage and are applied directly to ornamental plants, trees, field crops, cereals, and turf grasses to prevent crop damage from diseases such as leaf spot diseases, late blight/downy mildew, rice diseases, fruit rots, cereal seed-borne diseases, powdery mildews, rusts, and smuts [3]. It is common practice to apply a fungicide called azoxystrobin to the soil or use a systemic fungicide to protect crops from fungal infections. Wood-rotting fungus, yeasts, and filamentous fungi are all effectively combated by it [4]. It belongs to strobilurin family. Pesticide Action Network UK [5] notes that this patented strobilurin is now the most widely used fungicide in agriculture around the world. Over 85 crop types (including soybeans, rice, cereals, vegetables, fruit trees, and more) have been approved for use with it which has been shown by multiple studies [6, 7]. Under the brand name Amistar®, Azoxystrobin first appeared in the German market in February 1996 [8]. Several studies have shown deleterious effects of Azoxystrobin in fish animal model [9-11]. It affects metabolic processes by inhibiting complex III of mitochondrial respiratory system [12] but few studies have been conducted on the adverse impact of Azoxystrobin on mammalian

animal model therefore the present study has been planned to assess effect of Azoxystrobin on body weight, male reproductive organ weight and various biochemical parameters in testis of mice.

MATERIAL AND METHODS

Chemicals: Azoxystrobin (Methyl (2E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) was purchased from Rajlaxmi Pesticides Trading Co., West Bengal.

Experimental animals: Fifty-six adult male albino mice (*Mus musculus*) (approximately 8 weeks old) weighing between 30 to 35g were used for this study. Those animals were taken from animal house unit of IIS (deemed to be) University, Jaipur. The animals were housed in plastic cages, with six mice per cage. Floors of cages were covered with soft crushed wood shavings and provided with tap water *ad libitum* and fed with the standard commercial chow.

Experimental design

Mice were divided into seven groups and 6 animals were kept in each group. Group I was treated with vehicle (distil water for 30 and 60 days) and Group II, III, IV were treated with Azoxystrobin at three different doses i.e. 125, 250, 500 mg/kg body weight respectively for 30 days. Group V, VI, VII were orally administered with Azoxystrobin at dose level 125, 250,500 mg/kg body weight respectively for 60 days.

Body weight

The animals were weighed using the standard weighing balance at the beginning and at the end of the experiment. Initial and final body weight of all the animals was recorded and the change in the body weight of all the animals was calculated.

Organ weight

Testis, cauda epididymis, vas deferens and seminal vesicle of control and treated animal group were dissected out immediately after autopsy and weight of each organ was recorded using the standard weighing balance. The relative weight of each organ was calculated and changes in relative weight of each organ were analyzed in control and treated mice.

Biochemical parameters

For determination of biochemical changes in testes**,** the protein levels were measured by the method of Lowry *et al*., [13], the concentration of cholesterol was estimated by Liebermann-Burchard Reaction [14], Activity of alkaline phosphatase and Acid phosphatase was determined by the method given by Bessey *et al*., [15]. Succinate dehydrogenase activity was determined using method of Beatty *et al*., [16].

Statistical analysis

Statistical analysis was performed using SPSS software.

RESULTS

After 30 days of treatment with Azoxystrobin at different dose levels, body weight of mice decreased significantly only at high dose level while significant dose dependent decline was observed on body weight of mice when treated with different doses for 60 days. The percentage decline in body weight was noted as -9.51% in low dose, -14.38% in medium dose and -21.28% in high dose group after 60 days treatment. The deleterious effect of Azoxystrobin was somewhat ameliorated in the recovery group mice after withdrawal of treatment as evident from the body weight values which somewhat higher than 500 mg/kg body weight group but still less than control group indicating partial recovery in body weight. (Table 1). The percentage decline in organ weight for 30-day time period was non-significant in low and medium dose while significant ($p<0.05$) at high dose level (500 mg/kg b.wt). Changes in relative organ weight in the treated as well as control animals are given in Table 2. The testis, epididymis, vas deferens, seminal vesicles, kidney and liver weight of male mice treated with different doses of Azoxystrobin declined ($p<0.05$, $p<0.01$, $p<0.001$) in a dose dependent manner after 60 days treatment of low, medium and high dose respectively. Weight of testis, epididymis, Vas deferens, seminal vesicle, liver and kidney was found to be significantly (($p<0.05$) improved than high dose group while it was still significantly ($p<0.01$) lower than control group which indicates partial recovery.

Biochemical changes

Effects of Azoxystrobin on various biochemical parameters in testis are shown in Table 3. The Fungicide Azoxystrobin exposure did not reveal any significant effect on any biochemical parameters at low and medium dose treatment for 30 days while at high dose level, a significant change ($P<0.05$) was observed in biochemical status of testis. The total protein, Alkaline Phosphatase, Acid Phosphatase and activity of Succinate dehydrogenase content in testis of male mice treated with different doses of Azoxystrobin declined in a dose dependent manner after 60 days of treatment while cholesterol content was increased significantly in treated mice when compared with control mice. The deleterious effect of Azoxystrobin was somewhat ameliorated in the recovery group mice after withdrawal of treatment as evident from the

values which showed significant change in all the biochemical parameters when compared with high dose group but not up to the level of control group.

DISCUSSION

The incessant need to feed growing population has put tremendous pressure on the agriculturists and food scientists to maximize productivity and minimize agricultural losses due to various biotic and abiotic factors. This led to a wave for usage of chemical fertilizers, pesticides and insecticides, which although augment agricultural productivity, but are extremely maleficent for the environment as well as non-target animals like rodents, that visit these field. Considering this, the current study is focused on analyzing the maleficent effects of azoxystrobin, a fungicide that kills harmful fungi by inhibiting mitochondrial respiration by blocking electron transport chain [17]. The findings of this study showed that Azoxystrobin exposure might cause reproductive toxicity in male mice when exposed for a long duration as indicated by the results of present study azoxystrobin exposure for 60 days caused adverse impact on body and organ weight as well as on biochemical parameters in testis. Body weight changes and organ weight can be considered as an important toxicological end point to assess toxicity of chemicals. Azoxystrobin mediated decline in body weight may be attributed to decline in total protein content in mice as shown in previous results in this study. Similar results showing decline in body weight of rodents on exposure to fungicides and insecticides has also been shown in previous studies [18, 19]. Relative organ weight provides an accurate assessment of organ specific changes. Testes acts as main site of spermatogenesis. In addition to this, testes also synthesize testosterone, whose proper functioning maintains reproductive homeostasis. Epididymis acts as storage site for the sperms and aids in their maturation and finally transports them to vas deferens [20]. The secretions from seminal vesicle play a crucial role in survival and effective functioning of the sperms once they reach the female reproductive system. It forms 50-80% of the semen volume [21]. Therefore, the effect of azoxystrobin was monitored on weight of reproductive organs in mice which form an integral part of the male mice reproductive system and even a slight modulation in functioning of any of these organs severely compromises the male fertility [22, 23]. Reduction in the weight of male genital organs after treatment of Azoxystrobin might be correlated with low serum testosterone level as testosterone plays a major role in the regulation of structural and functional activities of testis and accessory sex organ. Similar results showing decline in weight of male reproductive organs in exposure to insecticides and fungicides in rodents have also been shown in previous studies [24-26]. Nevertheless, the deleterious effect of Azoxystrobin was somewhat ameliorated in the recovery group mice after withdrawal of treatment. This restoration of organ weight in recovery group after withdrawal of treatment may be attributed to re-establishment of metabolic homeostasis owing to quenching of treatment induced perturbation of HPG axis as well as re-establishment of oxidative equilibrium. In addition to the effect of azoxystrobin exposure on reproductive parameters, the researchers also analysed its effect on weight of two vital organs, kidney and liver that play a crucial role in maintenance of overall body homeostasis. Both kidneys and liver form an important part of the detoxification system, which helps in getting rid of xenobiotics and obnoxious toxicants accumulated in the body. Azoxystrobin mediated reduction in the weight of liver and maybe attributed to high level of ROS and RNS, which lead to induction of oxidative, and nitrosative stress followed by dismantling of oxidative equilibrium in rats. Azoxystrobin mediated decline in kidney and liver weight of mice is indicative of compromised renal and liver function. These results are in complete concordance with the previous set of results shown in this study, wherein, the exposure to azoxystrobin led to decline in protein content in mice. Reduced protein content maybe due to decreased liver activity, which play a key role in protein synthesis as well as protein degradation. Similar results have also been shown in a recent study by El-Hak *et al* [27], wherein exposure to azoxystrobin and acetamiprid led to impaired liver activity owing to treatment induced oxidative stress. However, the weight of kidney as well as liver was restored in recovery group of mice on withdrawal of azoxystrobin exposure, which is indicative of restoration of oxidative equilibrium in mice after withdrawal of treatment. A number of previous studies have demonstrated decline in kidney and liver function in response to treatment with EDCs [28, 29]. Proteins play an important role in maintenance of healthy body weight. Reduction in total protein content may occur due to Azoxytrobin mediated oxidative damage to the proteins, leading to protein denaturation as well as malicious effects of Azoytsrobin on functioning of sertoli cells, which play a key role in testicular protein synthesis. A number of previous studies have delineated the mode of action of pesticides/EDCs wherein exposure to exposure to pesticides causes' increased ROS production leading to oxidative burst. This further leads to induction of oxidative stress, which is the main perpetrator of many diseased conditions in the body. ROS free radicals oxidize proteins thus leading to decreased overall protein content. Furthermore, to add to the already anarchical situation, pesticides also produce RNS, which leads

to nitrosative stress. Both oxidative and nitrosative stress act in combination with each other and wreak havoc on the cellular machinery [30]. The results in this study show deleterious effect of fungicide Azoxystrobin on total protein content (mg/g) of testis of adult male albino mice treated with 125 mg/kg body weight, 250 mg/kg body weight, and 500 mg/kg of body weight respectively for 60 days. This decline in testicular protein content maybe attributed to increased proteolytic activity due to ROS. ROS triggers a cascade of malefic events leading to oxidative stress which further results in oxidation of proteins, hence, decreased total protein content. The research findings obtained in this study are in complete agreement with previous studies where too the researchers demonstrated decrease in protein content in response to exposure to EDCs [31, 32]. Cholesterol is an important biomolecule which performs many crucial functions like maintaining the integrity and fluidity of cell membranes and serve as precursor of substances that are critical for sustenance like bile acids, vitamin D and steroid hormones [33]. In testis, cholesterol performs two main functions: Providing structural integrity to plasma membrane and Precursor for testosterone biosynthesis. The cholesterol content in testis of male mice treated with different doses of Azoxystrobin increased in the 60-day time point in a dose dependent manner. The increase in cholesterol content in 30-day time point maybe attributed to underutilization of cholesterol owing to decreased testosterone biosynthesis. The restoration of the cholesterol levels after withdrawal of treatment may be attributed to re-instating of altered metabolic state and re-establishment of Hypothalamic-pituitary-testicular axis leading to utilization of cholesterol for testosterone biosynthesis. The results obtained in this study are in complete congruency with previous studies where too the researchers have demonstrated increase in cholesterol content in testis in response to exposure to different EDCs [34, 35]. Acid phosphatases and Alkaline phosphatases represent a class of lysosomal enzymes which play a crucial role in the process of spermatogenesis and sperm maturation. These enzymes are widely distributed throughout the testicular tissue with fewer lysosomes inside the mature sperms containing acid phosphatase vacuoles as well as in sertoli cells [36]. Both acid phosphatase and alkaline phosphatase are believed to be markers of membrane damage and tissue necrosis [37]. The results in our study showed that fungicide Azoxystrobin showed deleterious effect on Acid Phosphatase and Alkaline phosphatases content (IU/g tissue) of testis of adult male albino mice only at high dose level 30 days of treatment. The Acid Phosphatase and Alkaline phosphatases content in testis of male mice treated with different doses of Azoxystrobin declined in a dose dependent manner after 60 days treatment. Decrease in enzyme activity of Acid Phosphatase and Alkaline phosphatases in response to azoxystrobin maybe attributed to testicular degeneration characterized by leydig cell atrophy and shrinkage, disorganization of germ cells as well as decline in number of active spermatozoa in seminiferous tubules (results shown in histopathology and histomorphometry). All these factors were also accompanied by decline in sperm quality and motility. The deleterious effect of Azoxystrobin was somewhat ameliorated in the recovery group of mice after withdrawal of treatment as evident from the Acid Phosphatase and Alkaline phosphatases content values somewhat higher than high dose group but still less than control group. This restoration of acid phosphatase and Alkaline phosphatases content in recovery group after withdrawal of treatment may be attributed to re-establishment of metabolic homeostasis owing to quenching of treatment induced oxidative and nitrosative burst and re-establishment of testicular homeostasis and vasculature. A number of previous studies have also demonstrated decreased Acid phosphatase and alkaline phosphatase content in response to EDCs exposure in testis further leading to impaired spermatogenesis and overall decline in reproductive health and other biochemical parameters [38-40]. Testis harbors a number of mitochondrial enzymes, the ones involved in Glycolytic pathway, Krebs cycle as well as electron transport chain. Optimum activity of these enzymes is crucial for maintenance of testicular homeostasis. Both spermatogenesis and sperm maturation are high energy demanding processes and a slight perturbation in the activity of mitochondrial enzymes upsets the entire testicular bioenergetics thus adversely affecting spermatogenesis and debilitation in reproductive capacity. Exposure to EDCs causes induction of oxidative and nitrosative stress which disrupts the testicular mitochondrial dynamics and aberrant enzyme activity, thus affecting testicular health, spermatogenesis and other sperm quality parameters [41-45]. Results of present study showed deleterious effect of fungicide Azoxystrobin on Succinate dehydrogenase content (IU/g tissue) of testis of adult male albino mice treated with 125 mg/kg body weight, 250 mg/kg body weight, and 500 mg/kg of body weight respectively for 60 days. The succinate dehydrogenase content in testis of male mice treated with different doses of Azoxystrobin declined in a dose dependent manner in both the time points (30 days as well as 60 days). Decreased enzyme activity of succinate dehydrogenase in testis of azoxystrobin treated mice is indicative of disrupted energy metabolism in testis. Altered energy metabolism is a sign of testicular damage, which is clearly evident from histopathology and histomorphometric studies showing testicular degeneration characterized by

Leydig cell atrophy and shrinkage, disorganization of germ cells as well as decline in number of active spermatozoa in seminiferous tubules.

Table 1: Showing effect of Azoxystrobin on body weight of mice treated with three different doses for 30 and 60 days.

Body weight (g)	Duration (days)	Control	Azoxystrobin treatment (mg/kg b.wt)			Recovery
			Low dose (125 mg/kg b.wt)	Medium dose (250 mg/kg b.wt)	High dose (500 mg/kg b.wt)	
Initial	**30**	31.18±3.67	30.94±4.95	30.34±2.45	31.55±2.15	
Final		32.88±2.11 (5.45%)	29.85±2.45ns (-0.03%)	28.76±1.88ns (-0.05%)	28.21±3.66* (-10.58%)	
Initial	**60**	31.15±2.87	32.17±4.18	31.76±2.58	30.63±5.17	31.11±5.11
Final		34.21±3.15 (9.82%)	29.11±6.66* (-9.51%)	27.19±1.45** (-14.38%)	24.11±3.25*** (-21.28%)	26.34±2.35*a (-15.33%)

ns- Non significant from initial weight, *- significant at p<0.05 from initial weight, **- significant at p<0.01 from initial weight, ***- significant at p<0.001 from initial weight

(% change is from initial to final in similar group)

Table 2: Showing effect of Azoxystrobin on relative organ weight of mice treated with three different doses for 30 and 60 days.

Parameters	Duration (days)	Control	Dose of Azoxystrobin (mg/kg body weight)			Recovery (60 days)
			Lower dose (125 mg/kg b.wt)	Medium dose (250 mg/kg b.wt)	Higher dose (500 mg/kg b.wt)	
Testis (mg/100g b.wt.)	30	1221.65±12.18	1211.78±21.67ns	1208.72±12.34ns	1087.27±20.19*	
	60	1298.67±16.25	1191.54±17.81*	1067.22±21.56**	998.16±16.55***	1078±45.66**b
Epididymis (mg/100g b.wt.)	30	513.18±9.16	511.66±12.34ns	502.19±9.18ns	493.15±16.46*	
	60	561.56±22.18	546.68±16.13*	527.32±10.23**	511.18±24.66***	530.18±18.15**b
Vas deferens (mg/100g b.wt.)	30	157.25±9.11	154.18±11.98ns	151.66±7.67ns	141.87±21.56*	
	60	159.43±15.77	146.99±8.16*	137.18±11.26**	128.66±10.95***	141.88±16.56**b
Seminal vesicle (mg/100g b.wt.)	30	520.15±18.26	511.16±10.56ns	508.68±26.56ns	501.17±21.34*	
	60	521.13±17.65	508.46±8.75*	491.66±25.15**	486.17±22.56***	495.91±22.66**a
Kidney (mg/100g b.wt.)	30	582.27±11.64	578.72±10.35ns	571.24±20.11ns	564.46±20.16*	
	60	589.22±9.08	573.65±15.48*	543.22±8.65**	536.91±11.17***	547.85±19.34**a
Liver (mg/100g b.wt.)	30	2172.29±34.76	2166.54±41.64ns	2159.72±17.85ns	2147.85±34.56*	
	60	2176.11±45.16	2157.83±23.37*	2143.26±23.15**	2135.81±20.65***	2147.80±34.51**a

ns- Non significant from control, *- significant at p<0.05 from control, **- significant at p<0.01 from control, ***- significant at p<0.001 from control

a- significant at p<0.05 from highest dose at 60 days

b- significant at p<0.01 from highest dose at 60 days

Table 3: Showing changes in Biochemical parameters in the testis of mice treated with various doses of Azoxystrobin for the duration of 30 days and 60 days.

Biochemical parameters	Duration	Control	Low dose (125 mg/kg b wt.)	Medium dose (250 mg/kg b wt.)	High dose (500 mg/kg b wt.)	Recovery
Total protein (mg/g)	30 days	15.50 ± 0.764	14.83 ± 0.872^{ns}	13.00 ± 0.931^{ns}	11.17 ± 0.910 *	
	60 days	16.33 ± 0.715	13.17 ± 0.833*	11.50 ± 0.764**	10.00 ± 0.775***	12.67±0.47**b
Total cholesterol (mg/g)	30 days	0.275±0.010	0.288±0.006ns	0.296±0.008^{ns}	0.311±0.009*	
	60 days	0.331±0.013	0.353±0.012*	0.381±0.011**	0.398±0.015***	0.357±0.016*a
Alkaline phosphatase (IU/g tissue)	30 days	2.667±0.125	2.450±0.147$_{ns}$	2.183±0.107ns	2.083±0.149*	
	60 days	2.983±0.135	2.450±0.125*	2.217±0.119**	1.600±0.123***	2.36±0.152**b
Acid phosphatase (IU/g tissue)	30 days	3.550±0.152	3.133±0.180^{ns}	2.850±0.154^{ns}	2.217±0.144*	
	60 days	3.967±0.189	3.517±0.193*	2.900±0.152**	2.233±0.154***	2.682±0.257*a
Succinate dehydrogenase (pg formazan formed/mg protein/15 min)	30 days	17.50±0.764	16.00±0.775^{ns}	14.67±0.803^{ns}	13.67±0.760*	
	60 days	19.67±1.333	14.00±1.183*	11.67±1.022**	11.00±1.183***	13.65±0.74**a

Level of significance = Mean ± SE.M

^{ns} Represent non-significant * Represent p<0.05; ** Represent p<0.01; ***Represent p<0.001when treated groups compared with control group

^{a} Represent p<0.05; ^{b}Represent p<0.01; ^{c}Represent p<0.001 when recovery group compared with high dose group of 60 days)

CONCLUSIONS

The present study provides the first evidence that long term exposure of Azoxystrobin might cause adverse impact on male reproductive system by disturbing the level of enzymes and chemical required for synthesis of steroidogenic hormones and for maintaining sperm integrity in the testis. Therefore, excessive usage of Azoxytrobin should be avoided and more *in vivo* studies should be conducted to validate the impact of Azoxystrobin.

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