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Original article

REPRODUCTIVE AND GENOTOXIC EFFECTS OF DINOTEFURAN IN MALE SPRAGUE DAWLEY RATS

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Summary

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The objective of the current study was the evaluation of the genotoxic and reproductive effects of dinotefuran (DIN) insecticide in male Sprague Dawley rats. To achieve these objectives, forty male 10–12 weeks old Sprague Dawley rats were randomly divided into four equal groups; the first group was used as a control group; the other three groups were exposed to 40, 61, and 122 mg/kg body weight DIN by oral gavage for 8 weeks. Relative testicular weight, testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), malondialdehyde (MDA) and glutathione peroxidase (GPx) levels, sperm viability, sperm morphology, chromosomal aberrations assay, expression of interleukin 6 (IL-6) and cluster of differentiation 68 (CD68), and histopathological changes in testes of these rats were examined after 8 weeks. The results showed that DIN exposure resulted in a significant increase in testosterone, the percentage of dead and abnormal sperms, the percentage of metaphase cells with chromosomal aberrations, and expression of IL-6 and CD-68 but decreased GPx levels. Histopathological examination of the testes revealed hypertrophied seminiferous tubules of testes with a disrupted arrangement of germinal epithelial cells accompanied by many necrotic areas. It was concluded that exposure to DIN for 8 weeks induced hazardous impacts on the reproductive system and caused genotoxicity.

Key words: chromosomal aberrations assay, dinotefuran, gene expression

INTRODUCTION

Pesticides are synthetic chemicals that are used in agriculture to improve the yield of crops. Pesticide residues can remain for a long period and may affect many organisms in the environment (Yoneda *et al.*, 2018). A huge number of cases of death were detected in the world because of pesticide poisoning and most of these cases were in developing countries. The excessive using of pesticides leads to hazardous impacts on the exposed organisms. Neonicotinoids (NN) are synthetic insec-

ticides, that are used to control insects (Natalia & Robert, 2016) in many crops such as corn, soybeans, and other vegetation as fruits and vegetables (Cimino *et al.*, 2017). The spreading of NNs in the last 2 decades has been associated with infectious disease outbreaks in birds, fish, honey bees, and amphibians (Mason *et al.*, 2013). Dinotefuran (DIN) is an NN insecticide that belongs to the third generation of this group and has rapid and wide insecticidal action compared to other NNs. Moreover, it has agonistic action on the nicotinic acetylcholine receptors in insects (Yoneda et al., 2018). NNs induce reproductive toxicity, immunotoxicity, hepatotoxicity, and neurotoxicity (Fathy & Abdelkader, 2021) in animals and humans. However, there is insufficient data on the toxicity of DIN on the male reproductive system. Therefore this study intended to assess the reproductive and genotoxic effects of DIN in male Sprague Dawley rats, and this study was performed on rats in laboratory conditions in which environmental factors are under control. The reverse occurs in domestic animal experiments where environmental variables are not under control. Therefore, lab experiments facilitate the interpretation of data and allow investigation of the effects of DIN.

MATERIALS AND METHODS

Chemicals

A commercial formulation of DIN (Mitsui Chemicals Agro Group, INC., Japan) as a powder containing 20% (w/w) DIN as the active ingredient, colchicine (Oxford Lab Chem., India), sodium carbonate and potassium chloride (El Nasr Pharmaceutical Chemicals Co., Egypt), methyl violet, eosin, and nigrosin stain, methanol, glacial acetic acid, and Giemsa stain (S. D Fine-Chem Limited, India) were used. All chemicals and reagents have a high grade of purity.

Animals

Forty male Sprague Dawley rats (10–12 weeks old and weighing 120–150 g) were obtained from the Animal House, Faculty of Veterinary Medicine, Assiut University. Rats were housed for two weeks in plastic cages as an accommodation period before the experiment and remained in an environment with a temperature of 22±2.0°C and a 12-hour lighting cycle (light/dark). Water and feed were available all the time. Handling and care of laboratory animals were conducted according to the Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, according to The OIE standards for the use of animals in research under the No. 06/2023/0061.

Experimental design

Forty animals were separated into four groups of ten animals each:

1) Group 1: Control group, rats were given distilled water 1 mL/100 g by oral gavage day by day for 8 weeks;
2) Group 2: Rats were exposed to DIN (dissolved in distilled water) at a dose rate of 40 mg/kg body weight by oral gavage day by day for 8 weeks. This dose is 1/60 of the oral LD50 of DIN (2450 mg/kg b.w.) as reported by FAO/WHO (2013);
3) Group 3: Rats were administered DIN at a dose rate of 61 mg/kg body weight which was 1/40 LD50 by oral gavage day by day for 8 weeks;
4) Group 4: Rats were dosed DIN at a dose rate of 122 mg/kg body weight which represents 1/20 LD50 by oral gavage day by day for 8 weeks.

Samples collection

After 8 weeks, diethyl ether was used to euthanise 10 rats from each group. Blood samples were collected from the descending aorta in sterile tubes without anticoagulants and then centrifuged at 3000 rpm for 10 min to obtain serum. The serum samples were preserved at − 20 °C to estimate levels of testosterone, FSH, LH, MDA, and GPx activity. Sperm suspension was collected from the caudal portion of the epididymis with drops of normal saline at 37 °C to examine the vitality and morphology of sperms. Femoral bones were split up and the bone marrow was flushed for chromosomal aberrations assay. Samples of testes were divided into two parts; the first part was collected in RNA later solution for estimation of the gene expression of IL-6 and CD-68 and the second part was fixed in 10% formaldehyde for histopathological analysis.

Organosomatic index of testes

The total body weight and testicular weight were estimated and the testicular organ somatic index was calculated (testicular weight/ body weight).

Hormonal assays

According to the method of Tietz (1995), the concentration of serum testosterone was determined at a wavelength 450 nm using a testosterone ELISA kit (Biodiagnostic, Egypt) using an ELISA reader (Bio-Tec instrument). Serum FSH levels were estimated at 490–630 nm using an FSH ELISA kit (Biodiagnostic, Egypt) by ELISA reader (Bio-Tec instrument) according to Scott *et al.* (1989). Levels of serum LH were determined according to Beastall *et al.* (1987) at 490–630 nm using an ELISA kit (Biodiagnostic, Egypt) on Bio-Tec instrument ELISA reader.

Estimation of serum MDA and GPx activity

MDA level was measured at 534 nm according to the method of Ohkawa *et al.* (1979); and GPx activity was estimated according to the method of Paglia & Valentine (1967) at 340 nm using commercial kits (Biodiagnostic, Egypt) on UV-2100 spectrophotometer (Unico, USA).

Epididymal sperm parameters

Sperm viability test. The cauda epididymal duct was incised, and then the semen was diluted with saline. Twenty µL of sperm suspension was mixed with 20 µL of 0.05% eosin and 50 µL of nigrosin in a test tube. The stained sperm mixture was smeared on slides. In each slide, two hundred sperms were examined by a light microscope (Olympus BX 43, Japan). The head of dead sperms appeared pink and live sperms were not stained (Wyrobek *et al.*, 1983).

Sperm morphology test. The percentage of abnormal sperm was estimated according to the method of Menkveld (2010). The sperm sample was diluted with 1% sodium chloride solution then sperm suspension was smeared on a slide and dried in the air. The slides were stained with a mixture of one part 1% sodium carbonate solution with nine parts 1% methyl-violet solution. After 4 to 5 min, the slides were washed with distilled water then dried with filter paper and sperms were examined under a light microscope (Olympus BX-43, Japan).

Chromosomal aberrations assay. The percentage of chromosomal aberrations in bone marrow cells was determined according to Preston *et al.* (1987). Briefly, colchicine (4 mg/kg b.w.) was administered intraperitoneally 2 h before the collection of the bone marrow cells. The rats were euthanised by cervical dislocations

then femur bones were cut and the bone marrow was flushed in 0.56% KCL. The collected cells were incubated at 37 °C for 30 min and then centrifuged for 10 min at 1,000 rpm. Cells were fixed in a fixative consisting of 3 parts methanol and one part acetic acid then smeared on clean dry slides. The smeared slides were stained with 5% Giemsa solution for 15 min. One hundred well-spread metaphases were examined by light microscopy (Olympus BX-43, Japan) at a magnification of ×100 for each group. Several types of CAs such as chromatid breaks (CB), chromatid gaps (CG), centromeric association (CA), chromatid fragments (CF), ring formation (RF), centromeric attenuation, deletion, and polyploidy were recorded.

IL-6 and CD68 mRNA expression

Total RNA was extracted from tissue lysate with Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA) and then its quantity and quality were estimated on Beckman dual spectrophotometer (USA). Reverse transcription of extracted RNA was performed by SuperScript IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) followed by PCR. Real-time quantitative PCR (Step One Applied Biosystem, Foster City, USA) was used for the analysis of IL-6 and CD68 mRNA expression.

Primers sequences of all studied genes were as follows:

- IL-6: forward sequence: TACCACT TCACAAGTCGGAGGC; reverse sequence: CTGCAAGTGCATCATCG TTGTTC; NCBI reference sequence: NM_031168;
- CD68: forward sequence: GGCGGT GGAATACAATGTGTCC; reverse sequence: AGCAGGTCAAGGTGAA CAGCTG; NCBI reference sequence: NM_009853;
- GAPDH: forward sequence: CAT CACTGCCACCCAGAAGACTG; reverse sequence: ATGCCAGTGAG CTTCCCGTTCAG; NCBI reference sequence: NM_008084.

Data in the RT-PCR were expressed as cycle threshold (Ct) values. The relative quantification (RQ) of each target gene was quantified and normalised to the housekeeping gene according to the calculation of delta-delta Ct (ΔΔCt) and the RQ of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Histopathological investigation

Fresh specimens from the rat testes from all experimental groups were freshly harvested and fixed in 10% neutral buffered formalin. Tissue samples were processed routinely, sectioned at 4 µm thickness, and stained by haematoxylin and eosin. Histopathological analysis was performed by light microscopy (Olympus, CX31; Tokyo Japan) (Suvarna *et al.*, 2018).

Statistical analysis

The results were presented as mean ± SE. One-way analysis of variance (ANOVA) followed by a post-hoc test (Tukey) was applied to detect significant differences among groups. The analyses were performed using the SPSS program for Windows, version 16.0 (Borenstein *et al.*, 1997). Differences between the groups were significant at $P \leq 0.05$.

RESULTS

The relative testicular weight did not show significant changes in the exposed groups in comparison with the control group (Table 1).

Table 1. Testicular organosomatic index in rats after oral administration of DIN (40, 61, and 122 mg/kg/b.w.) for 8 weeks. Values were expressed as means ± SE (n = 10).

Groups	Testicular organo-somatic index
Control	2.13 ± 0.21
40 mg/kg DIN	2.11 ± 0.12
61 mg/kg DIN	2.10 ± 0.07
122 mg/kg DIN	1.83 ± 0.00

The levels of FSH and LH were not significantly different in the exposed groups in comparison with the control group while testosterone level demonstrated a significant (P<0.05) increment in the 122 mg/kg dose group as compared with the controls (Fig. 1).

The level of MDA did not show significant changes in the exposed groups vs the control group whereas GPx activity illustrated a significant (P<0.01) decrease in all exposed groups. However, GPx level in rats exposed to 122 mg/kg DIN showed a significant (P<0.05) increase when compared to groups exposed to 40 and 61 mg/kg (Table 2).

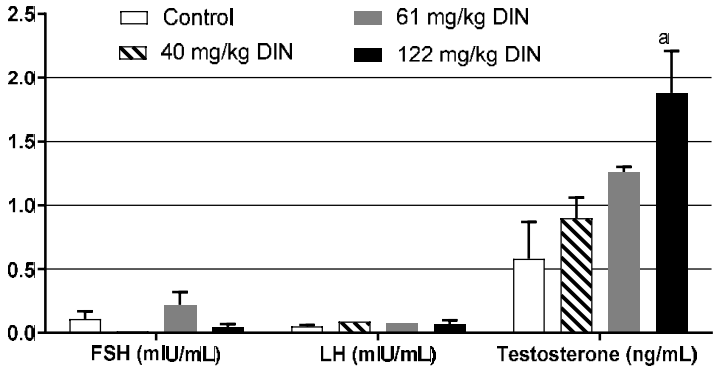

Fig. 1. Testosterone, FSH, and LH hormones levels in rats after oral administration of DIN (40, 61, and 122 mg/kg/b.w.) for 8 weeks; a: P≤0.05 vs the control group.

Table 2. MDA level and GPx activity in rats exposed to 40, 61, and 122 mg/kg of DIN for 8 weeks. Values were expressed as means ± SE (n = 10).

Groups	MDA (nmol/mL)	GPx (U/L)
Control	4.20±0.00	419.75±0.11
40 mg/kg DIN	4.73±0.29	357.55±1.41 a
61 mg/kg DIN	5.27±0.12	359.50±3.17 ac
122 mg/kg DIN	4.73±0.46	371.05±2.91 abc

a: P≤0.05 vs the control group; b: P≤0.05 vs the 40 mg/kg group; c: P≤0.05 between 61 and 122 mg/kg groups in each column.

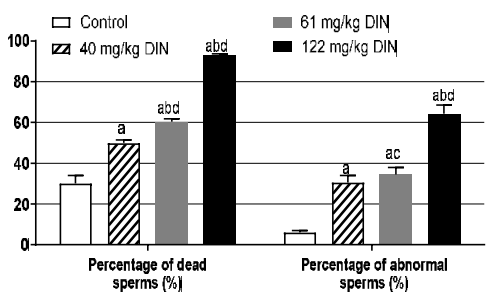

Fig. 2. Sperm viability and morphology in male rats exposed to 40, 61, and 122 mg/kg b.w. DIN for 8 weeks; a: P≤0.05 vs the control group; b: P≤0.05 vs 40 mg/kg group; c: P≤0.05 between 61 and 122 mg/kg groups.

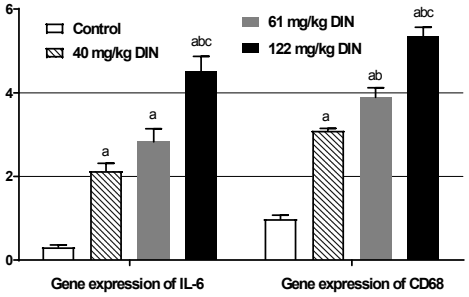

Fig. 3. Gene expression of IL-6 and CD68 in testes of rats exposed to 40, 61, and 122 mg/kg DIN for 8 weeks; a: P≤0.05 vs the control group; b: P≤0.05 vs 40 mg/kg group; c: P≤0.05 between 61 and 122 mg/kg groups.

Table 3. Percentage (%) of chromosomal aberrations in bone marrow cells of rats exposed to 40, 61 and 122 mg/kg DIN for 8 weeks. Values were expressed as means ± SE (n=10).

Group	Control	40 mg/kg DIN	61 mg/kg DIN	122 mg/kg DIN
Normal meta-phase cells	89.00±0.96	61.02±1.40 a	50.00±2.62 ab	31.01±1.18 abc
Chromatid break	0.98±0.98	1.99±0.99	6.03±1.77	9.00±1.75 ab
Chromatid frag-ment	3.00±0.03	12.00±1.75 a	12.95±2.50 a	12.98±0.86 a
Ring formation	1.01±1.01	2.02±1.01	7.04±2.69	12.95±2.50 ab
Deletion	1.99±0.99	6.00±0.05	7.93±3.49	12.00±1.75 b
Centromeric asso-ciation	0.00±0.00	3.98±1.99	4.99±0.98a	3.00±0.02
Centromeric at-tenuation	3.00±.029	4.99±0.98	6.00±1.75	9.03±1.80 a
Polyploidy	1.01±1.01	7.99±0.96	5.05±3.64	10.01±1.05a

a: P≤0.05 vs the control group; b: P≤0.05 vs the 40 mg/kg group; c: P≤0.05 between 61 and 122 mg/kg groups in each row.

In this study, the percentage of dead sperms showed a significant (P<0.01) increase at 40, 61, and 122 mg/kg groups as compared with the control group. In addition, 61 mg/kg (P<0.01) and 122 mg/kg (P<0.05) DIN groups revealed a significant increase in this parameter than the 40 mg/kg group. Also, there was a significant (P<0.01) increase in the 122 mg/kg DIN group in comparison with the 61 mg/kg group (Fig. 2). The percentage of abnormal sperms was significantly higher in groups exposed to 40 (P<0.05), 61 and 122 mg/kg (P<0.01) as compared to the control group. In addition, the 122 mg/kg DIN group marked a significant (*P*<0.01) increase vs both the 40 and 61 mg/kg groups (Fig. 2).

The percentage of metaphase cells with chromosomal aberrations was significantly (P<0.01) increased in 40, 61, and 122 mg/kg DIN groups as compared to the control group. This increase was significant (P<0.01) in the 61 and 122 mg/kg groups as compared to the 40 mg/kg group. There was also a significant increase (P<0.01) between the 122 mg/kg and 61 mg/kg groups. The percentage of CB was significantly higher in
the 122 mg/kg dose group as compared to the control (P<0.01) and 40 mg/kg (P<0.05) dose groups. The CF percentage showed significant (P<0.01) increments in 40, 61, and 122 mg/kg dose groups vs control rats. RF percentage was significantly (P<0.01) increased in the 122 mg/kg group as compared to both the control and 40 mg/kg dose groups.

In the 122 mg/kg group, the percentage of deletion revealed a significant (P<0.05) increase compared to the 40 mg/kg dose group. The CA percentage in the 61 mg/kg group was significantly higher (P<0.05) than controls. The percentage of centromeric attenuation and polyploidy were significantly (P<0.05) increased in the 122 mg/kg group compared to non-exposed rats (Table 3).

In this study, the gene expression of IL-6 presented a significant increase (P<0.01) in 40, 61, and 122 mg/kg groups as compared to the control group. The increase in the 122 mg/kg group was also significant (P<0.01) than values in the 40 and 61 mg/kg groups. Concerning the gene expression of CD-68, it was statisti-

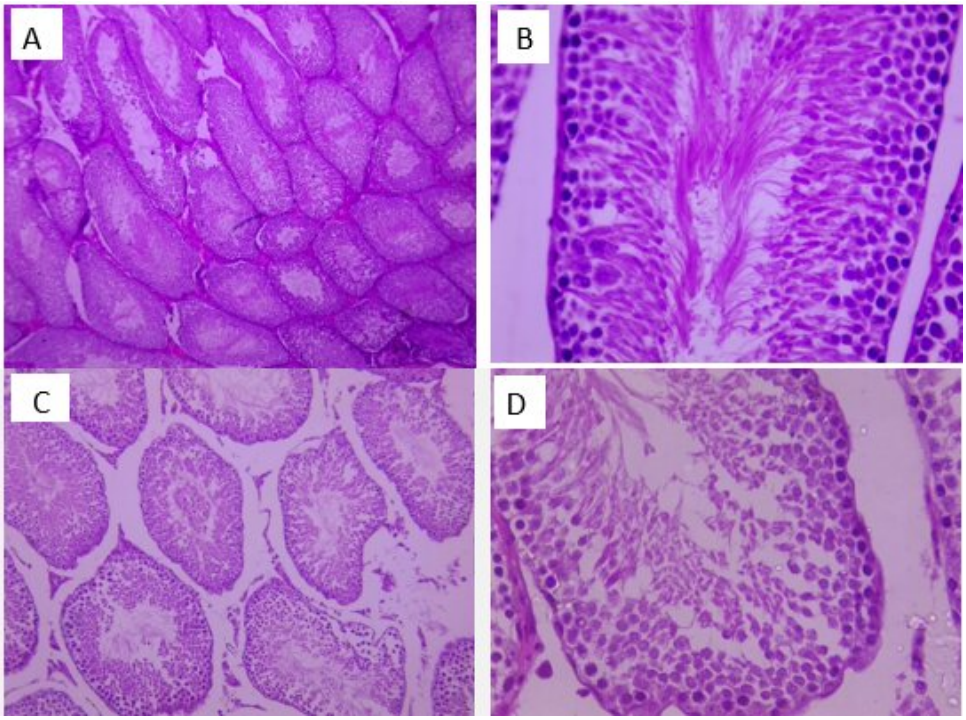

Fig. 4. Histopathological examination of testes in the control group (**A, B**) showing the normal structure of the seminiferous tubules; 40 mg/kg DIN dose group **(C, D)** showing hypertrophied ST with a disrupted arrangement of germinal epithelial cells (Magnification ×10 in A & C and ×40 in B & D).

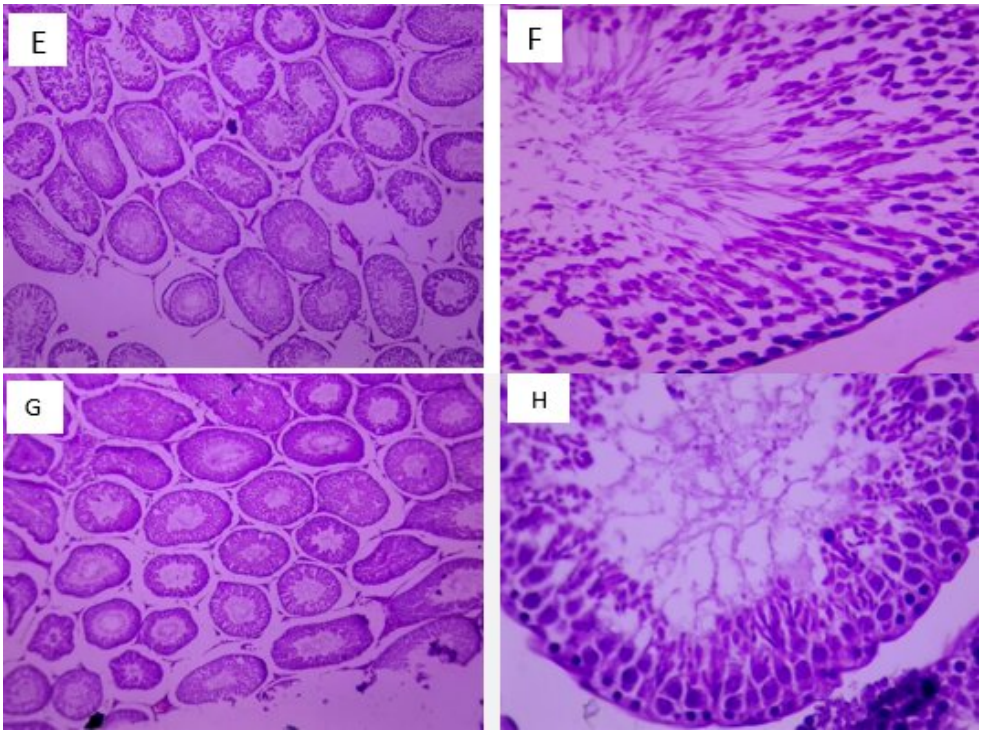

Fig. 4 (cont'd). Histopathological examination of testes in the 61 mg/kg DIN dose group **(E, F)** showing an irregular arrangement of spermatogenic epithelial cells accompanied by many degenerative areas. 122 mg/kg DIN dose group **(G, H)** showing shrinkage of the ST lining epithelium accompanied by many necrotic areas and apoptosis of many spermatids. (Magnification ×10 in E & G and ×40 in F & H).

cally significantly higher in 40, 61 and 122 mg/kg groups vs controls (P<0.01). Furthermore, the 61 (P < 0.05) and 122 mg/kg (P < 0.01) groups differed significantly as compared to the 40 mg/kg group; and the CD-68 expression in 122 mg/kg group was significantly different (P<0.01) than that in the 61 mg/kg group (Fig. 3).

Histopathological changes in all DIN-exposed groups are presented on Fig. 4. The testes of the control group showed the normal structure of seminiferous tubules lined by normal germinal epithelium composed of maturing germ cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) (Fig. 4A, B).

Histopathological changes in the testes of rats exposed to 40 mg/kg DIN showed hypertrophy of seminiferous tubules with a disrupted arrangement of germinal epithelial cells (Fig. 4 C,D). Similar findings were observed in sections from groups exposed to 61 and 122 mg/kg DIN doses with an apparent damaging effect in the 122 mg/kg DIN group compared to the other groups. Exposure to 61 mg/kg DIN induced an irregular arrangement of spermatogenic epithelial cells accompanied by many degenerative areas (Fig. 4E, F). These sections from the 122 mg/kg

DIN group indicated shrinkage of lining epithelium of the seminiferous tubules accompanied by many necrotic areas and apoptosis of many spermatids and degenerative spermatozoa (Fig. 4G, H).

DISCUSSION

Utilisation of pesticides in agriculture has increased (Al-Ghanim, 2012) leading to their persistence in the environment for a long time (Boivin *et al.*, 2005) and the occurrence of potential health hazards such as neurological, respiratory, reproductive diseases and cancers (Zhou *et al.*, 2019). The current study was conducted as a laboratory experiment as this type of experiment gives relatively "clean" data while field experiments yield data "noise" from uncontrolled factors (Calisi & Bentley, 2009). FSH, LH, and testosterone hormones have main roles in spermatogenesis and are very crucial for the function of the testes (Holdcraft & Braun, 2004); therefore serum levels of these hormones were estimated. In our study, testosterone levels indicated a significant increase in the 122 mg/kg DIN group when compared to the control group. This is in contrast with another study conducted by Mohamed *et al.* (2022) who found no significant change in testosterone level in male Sprague Dawley rats after exposure to sulfoxaflor at a dose rate of 25, 100, and 500 mg/kg b.w. for 4 weeks. However, exposure of rats to acetamiprid at a dose of 12.5, 25, and 35 mg/kg for 90 days led to a significant decrease in testosterone levels. In this study, the increase in testosterone level in the group exposed to 122 mg/kg may result from DIN-induced oxidative stress in Leydig cells, and disturbance in testosterone secretion (Mosbah *et al.*, 2018). However, testosterone is synthesized from cholesterol and the increased level of testosterone may be due to increment in cholesterol level (Yilmaz *et al.*, 2018).

In the present study, GPx activity revealed a significant decrease in all exposed groups as compared to controls. This is in agreement with Zhang *et al.* (2011) who found that GPx was reduced when mice were exposed to acetamiprid at a dose of 30 mg/kg. Oral administrations of imidacloprid at a dose rate of 16.9 mg/kg in male Wistar rats led to declined GPx activity (Mahajan *et al.*, 2018). Glutathione contains an active thiol group in the form of a cysteine residue, it acts as an antioxidant either by scavenging reactive oxygen species and electrophiles or as a cofactor for many enzymes (Lushchak, 2012). In the current study, the reduction in the activity of GPx may be due to an increase in the production of reactive oxygen species in DIN toxicity. The percentage of dead and abnormal sperms showed a significant increase at 40, 61, and 122 mg/kg DIN groups as compared with the control group. This corroborates with Saber *et al.* (2021) who revealed that exposure of rats to imidacloprid at a dose of 22.5 mg/kg led to a significant decline in sperm count, motility, and viability with a marked elevation in the percentage of sperm morphological abnormalities as compared to the control group. Also, the treatment of adult rats with imidacloprid for 60 days led to severe damage to testicular tissue and sperm vitality (Arıcan *et al.*, 2020). The percentage of dead and abnormal sperms increased after exposure to DIN because of the reduction of antioxidant enzymes and generated free radicals which led to oxidative damage in the cellular membranes of spermatozoa (Bal *et al.*, 2012).

In the current study, the percentage of metaphase cells with chromosomal aberra-

tions was significantly increased at 40, 61, and 122 mg/kg DIN groups as compared to the control group. This finding is in line with data of Bagri *et al.* (2016) indicating that daily exposure to imidacloprid at a dose rate of 22 mg/kg b.w. for 28 days in mice increased significantly chromosomal aberrations as compared to the control group. The exposure to imidacloprid at a dose rate of 22.5 mg/ kg b.w. for 4, 8, and 12 weeks resulted in elevated chromosomal aberrations percentage by the 8th and 12th weeks of exposure (Mohamed *et al.*, 2020). The presence of an electronegative pharmacophore (nitroimine N-substituent ¼NNO2) in the nitroguanidine moiety of neonicotinoids such as DIN, causes binding to the bases of DNA and forming adducts leading to DNA strand breaks (Tomizawa & Casida, 2005). The reasons for structural chromosomal aberrations are direct DNA damage, replication of damaged DNA templates, and inhibition of DNA synthesis (Kataria *et al.*, 2016).

In this study, the significantly increased IL-6 and CD-68 gene expression in 40, 61, and 122 mg/kg DIN groups corresponds with results of Tigges *et al.* (2022) that exposure of adult rats to parathion and paraoxon led to an increment in IL-6 expression. Also, IL-6 expression increased by exposure of rainbow trouts to hydrocarbons and chlorpyrifos (De Anna *et al.*, 2021) and exposure of pre-pubertal rats to 0.7 g/kg mono-2-ethylhexyl phthalate increased significantly the expression of inflammatory CD-68. The increased expression of IL-6 and CD-68 in our results revealed the occurrence of inflammation by DIN.

Histopathological investigation of testes, demonstrating hypertrophied seminiferous tubules with a disrupted arrangement of germinal epithelial cells in the 40 mg/kg group, irregular arrangement of spermatogenic epithelial cells accompanied by many degenerative areas in the 61 mg/kg group and shrinkage of the lining epithelium of seminiferous tubules accompanied by many necrotic areas and apoptosis of many spermatids and degenerative spermatozoa after exposure to 122 mg/kg DIN were similar to the results of Zakzook *et al.* (2020) who revealed that exposure to dimethoate induced degeneration, necrosis, and loss of the spermatogenic cell layer in testes. In addition, subchronic exposure of male rats to acetamiprid induced a decrease in the number of spermatogenic germ cells damaged spermatogenic cells with vacuole formation in seminiferous tubules (Arıcan *et al.,* 2020).

CONCLUSION

Exposure to DIN increased the blood level of testosterone hormone and decreased GPx activity. Also, it increased the percentage of dead and abnormal sperms, the percentage of metaphase cells with chromosomal aberrations, and gene expression of IL-6 and CD-68. In addition, histopathological examination revealed hypertrophy of the lining epithelium of seminiferous tubules accompanied by many necrotic areas. In conclusion, exposure of rats to DIN for 8 weeks induced adverse effects on the reproductive system and caused genotoxic effects.

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