Egypt. J. Chem. **Vol. 65**, No. 11 pp. 677 - 689 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Biochemical and histopathological evaluations of thiamethoxam on the male reproductive system

Entsar R. Abd-Allah[1], Abd-Elwahab El-Ghareeb[2], Olfat S. Hafez[2], Heba A. Abd El-Rahman[2]*

[1]Department of Zoology, Faculty of Science, Al-Azhar University, Nasr City, Egypt

[2]Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt

Abstract

Neonicotinoid insecticides usage is currently widespread, but this poses a challenge when considering the potential for occupational and environmental contamination. One of the most extensively used insecticides is thiamethoxam (TMX), a second-generation neonicotinoid insecticide. This study aimed to see if sub-lethal dosages of TMX insecticide had any negative impacts on epididymal sperm parameters, serum hormones, oxidative status, and testicular histology. The experimental cohorts were given a low dose of TMX (156 mg/kg bw), a high dose of TMX (312 mg/kg bw), or an untreated control for eight weeks. The sperm count, percent of viability, motility, and motility progressing and fructose level significantly decreased in both TMX-treated groups compared to the control group. Furthermore, TMX administration induced sperm morphological defects, serum hormone disturbances as significant reduction testosterone level, oxidant/antioxidant status imbalance as significant decline in catalase (CAT) and glutagthione peroxidase (GSH) content and significant rise in malondialdehyde (MDA) level and testicular histopathological alternation. TMX caused significant increase DNA damage in testicular tissue represented in tail DNA percent, comet percent, comet length, tail moment, and Olive moment. In conclusion, TMX exposure may have a deleterious impact on male albino rats' fertility through spermatogenesis, steroidogenesis and testicular redox status disruption, and testicles DNA impairment.

Keywords: Thiamethoxam, sperm quality, histopathology, reproductive hormones, oxidative stress, DNA damage, male Wistar rats

1. Introduction

Infertility is defined by the European Society for Human Reproduction and Embryology (ESHRE) as the failure of a pregnancy to occur within two years following normal coital openness [1].

Pesticides, according to the World Health Organization, are compounds used to kill pests such as bugs, rodents, growths, and unwanted vegetation [2]. Pesticides affect the male reproductive system in a variety of ways, affecting DNA structure and producing gene mutations that can lead to birth abnormalities or infertility. Epigenetic effects are changes in the way genes are expressed. They have the potential to function as synthetic endocrine disruptors [3]. Pesticide exposure results in the formation of Reactive Oxygen Species (ROS), which include free radicals with unpaired electrons, such as the Hydroxyl Radical (•OH), Superoxide Anion (O_2•), as well as non-free particles, such as singlet oxygen ($1O_2$) and Hydrogen Peroxide (H_2O_2). Because there are so many unpaired electrons in the particle, it is extremely sensitive [4].

The most widely used insecticide class is neonicotinoids and [5] defined neonicotinoids as a class of neuroactive insecticides that are structurally similar to nicotine. They are presently among the most extensively used pesticides in Egypt, owing to their great preference for invertebrate creatures over vertebrate species [6]. N-nitroguanidines (imidacloprid, thiamethoxam, dinotefuran, and clothianidin) and N-cyanoaminides (acetamiprid and thiacloprid) are two types of insecticides. They bind to nicotinic acetylcholine receptors (nAChRs) in sucking bugs and influence the beginning of electrical signals in the postsynaptic neuron. The presence of an electron-withdrawing group, such as cyano or nitro, is a crucial structural feature of these insecticides, as it contributes to their selectivity. These pesticides have insect-specific toxicity rather than mammalian toxicity. This specificity is what allows neonicotinoid mammalian toxicity to be

*Corresponding author e-mail: hebaabdelrahmana15@cu.edu.eg; (Heba A. Abd El-Rahman).

Receive Date: 06 February 2022; Revise Date: 26 February 2022; Accept Date: 28 February 2022.

DOI: 10.21608/EJCHEM.2022.120399.5400.

reduced [7]. In male bumblebees, neonicotinides have been shown to reduce sperm viability and living sperm quality [8].

The formation of free radicals by certain neonicotinoids has been linked to oxidative stress [9]. Furthermore, these insecticides may have negative effects on a variety of organs, including the brain [10], the testis [11], and the heart [12].

TMX is a second-generation neonicotinoid that binds more tightly to creepy crawly nicotinic acetylcholine receptors (nAChRs) than to those of well-developed organisms [8]. The efficacy of TMX at low dosages, the variety of administration modalities, and the long half-life is all reasons for its widespread use [14]. Desulphuration to the equivalent Oxon derivative by cytochrome P450 enzymes activates it [15]. TMX has a strong insecticidal action on a wide range of economically important pests. On the other hand, the administration of TMX to experimental animals had a significant negative impact on the function of several organs [16]. Oral administration of TMX reduced plasma cholesterol levels, an early indicator of liver failure that is likely linked to future histopathological alterations, and boost protooncogenes, induction of apoptosis, and tumor progression in mice [17-18]. [19] Found that rats given different dosages of TMX had an increase in anxiety behavior and a substantial decrease in both high-affinity choline uptake (HACU) and acetylcholinesterase activity in different brain areas. According to [20], TMX is a very efficient systemic and contact insecticide with a relatively large oral LD50 in albino rats (1563 mg/kg bw), indicating little acute mammalian toxicity.

When a substantial amount of TMX is consumed, it can cause serious poisoning, with the reproductive system being the primary target [21]. The effects on male fertility may damage spermatozoa directly, alter Sertoli cell or Leydig cell function, or disrupt endocrine function at any stage of hormonal regulation (hormone synthesis, release, storage, transport, and clearance; receptor recognition and binding; thyroid function; and the central nervous system [22]. Chemical alterations in sperm nuclear proteins (protamines), which interact with DNA during the last stages of spermatogenesis, are one of the factors that contribute to male reproductive toxicity [23].

It has the potential to harm mammalian reproductive well-being. In vitro, TMX triggers lipid peroxidation (LPO) as malondialdehyde (MDA), alters antioxidant enzyme performance, and impairments DNA [24]. Lessened water invertebrate growing and reproduction, decreased egg development, postponed breeding age, undesirable psychological alterations, and ovarian disruption organization can all be caused by sub-lethal doses [25].

In rats, single oral dosages of TMX are swiftly and practically completely absorbed. It is broadly dispersed throughout the body, with the liver having the largest tissue residues. At the highest dose level (100 mg/kg bw), TMX is poorly metabolized in rats, with 70-80 percent of the dose being excreted unaltered, in contrast to complete metabolism at the lowest dose level. The breaking of the oxadiazine ring to create the nitroguanidine CGA322704 is the most important biotransformation (clothianidin) [26]. The N-demethylated nitroguanidine metabolite CGA265307 can be produced directly from clothianidin or via an intermediary N-demethylated thiamethoxam metabolite (CGA330050). Because it can produce more formaldehyde than any other commercial neonicotinoid, the production of formaldehyde is thought to be an additional route for TMX-induced hepatotoxicity and hepatocarcinogenicity [27].

2. Aim of the Work

In view of the foregoing information, there is growing worry about TMX's unusual potentially adverse effects, as well as a significant need to investigate TMX conceptive toxicity in rodents. The purpose of our study was to explore the toxic effect of TMX on the reproductive health of adult Wistar male rats.

3. Material and Methods

3.1. Chemical and kits

The Mammalian &Aquatic Toxicology Department, Agriculture Research Center (ARC), Giza, Egypt, provided a commercial formulation of TMX, the chemical name of which is TMX3-(2-chloro-1,3-thiazol-5-ylmethyl-5-methyl-1,3,5oxadiazinan-4-ylidene(nitro)amine. Hormones produced by (Sunlong biotech co, LTD). Kits of oxidative indicators obtained from a bio diagnostic Company (Egypt).

3.2. Laboratory animals

Twenty-one male albino rats (Rattus norvegicus) were used in the current trail research. Male albino Wistar rats, sexually mature and weighing 170-180 g, were procured from the animal house of National organization for drug control and research (NODCAR). They were kept in a microbe-free environment with conventional temperatures (23±1˚C), relative humidity (55±10%), and a 12/12 hr light/dark cycle, and given a standard pellet diet and water.

3.3. Ethic consideration

All experimental modification and treatment followed ethical and human research norms. The research was authorised by Cairo University's Faculty of Science's Institutional Animal Care and Use Committee (CU/IF 83/19).

3.4. Study groups

Thiamethoxam was dissolved in tab water and given to rats orally (4.1 mg in one Litter). The rats were divided into three groups, each of which had seven rats. The trial lasted eight weeks (56 days), which corresponds to the completion of spermatogenesis [28]. The groups were as follows:

Group (1): received water along period of the experiment (the control group)

Group (2): received TMX insecticide orally at low dose 156 mg/kg bw (1/10 oral LD50).

Group (3): received TMX insecticide orally at high dose 312 mg/kg bw (1/5 oral LD50).

The animals were dissected and exposed testis with epididymis and seminal vesicles after being anaesthetized with sodium pentobarbital (IP dosage) at the end of treatment. The tissue was washed with normal saline, and weighted.

3.5. Serum preparation

Fill clean tubes with entire blood and no anticoagulant. Tubes are centrifuged for 20 minutes at 3,000 rpm after being incubated at room temperature for 10-20 minutes. For hormonal analysis, the sera were collected and kept at -20°C.

3.6. Serum hormone analysis

The serum from all groups was tested for testosterone (T), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels using ELISA kits from Sun Long Biotech Co., LTD. Sandwich-ELISA is the method used in this ELISA kit. This kit includes a Microelisa stripplate that has been percolated with a hormone-specific antibody. The appropriate Microelisa stripplate wells are filled with standards or samples, which are then mixed with the specific antibody. Then, in each Microelisa stripplate well, a Horseradish Peroxidase (HRP)-conjugated antibody specific for hormone is introduced and incubated. The components that aren't needed are rinsed away. Each well receives the TMB substrate solution. Only the wells containing hormone and HRP conjugated hormone antibody will appear blue before turning yellow after the stop solution is added. At a wavelength of 450 nm, the optical density (OD) is determined spectrophotometrically. The OD value is proportional to the hormone level. By comparing the OD of the samples to the standard curve, you may compute the hormone levels in the samples.

	Testosterone	LH	FSH
Precision	CV (%) = SD/meanX100 Intra-Assay: CV<10% Inter-Assay: CV<12%		
Assay range	0.1-3.8 ng/ml	0.1-7 IU/L	0.5-10 IU/L
Sensitivity	0.01 ng/ml	0.01 IU/L	0.1 IU/L

3.7. Sperm motility assessment

Sperm motility was observed by light microscopy (40X) in 10 fields of view. Every rat's right caudal epididymis was washed and finely minced in pre-warmed normal saline (37 °C). Motility was measured by counting at least 100 sperm from five separate regions and calculating the percentage of motile sperm, and motility progression percentage (sperm that are swimming in a regularly straightforward route). Sperm motility was evaluated by the method of Morrisey [29]. The sperm were counted using a hemocytometer following the methodology [30-31].

3.8. The resazurin reduction (RRT)

The ability of metabolically active sperms to turn the resazurin dye (blue) to resorufin (pink) is used in this assay. The RRT proportion is the most closely linked to sperm motility, count, morphology, and viability [32].

3.9. Fructose in Semen

High fructose levels in sperm are common, and this sugar is almost exclusively derived from the seminal vesicles. The quantity of fructose in human sperm and the fructolysis indices were measured to investigate if there was a correlation between two glycolysable sugars and sperm concentration. The resorcinol method was used to assess the amount of fructose in seminal plasma. Fructose interacts with resorcinol in a strong hydrochloric acid (HCl) solution to generate a crimson chemical. At a wavelength of 560 nm, compare the coloric combination of Zinc and Fructose to blanks [33].

3.10. Determination of Sperm morphology and viability

Aliquot 10 ul sample was placed on a slide, distributed with another slide, and then allowed to dry. Eosin (1% Eosin and 10% Nigrosin) stain was used to stain the dried samples. This dye can pass through the sperm membrane. Dye route is prohibited if the membrane is intact, as it is in the case of live spermatozoa. The dye will flow into the cytoplasm of the sperm if the membrane is disrupted, which happens frequently with dead or dying spermatozoa [34-35]. The incidence of total anomalies was reported, and imprecise form and conformation of either the head or the tail were regarded abnormal.

3.11. Evaluation of oxidative status

Each rat's left testis was removed and stored at -20°C in a clean Eppendorf tube for biochemical analysis. Freeze testes were homogenized in PBS (1:10 ml) using a mixing device for tissue homogenate preparation. The solution was centrifuged at 4000 rpm for 20 minutes, and the supernatant residue was utilized to determine MDA using method of Satoh [36], GSH based on Beutler et al. [37] method, and CAT as described by Aebi [38].

3.12. Evaluation of histopathology

The right testis was fixed in 10% formalin and prepared for paraffin block construction; sequential five-micron thick slices were cut and stained with hematoxylin and eosin (H&E) for regular histological examination [39]. The slides were examined using an AmScope light microscope, and the photos were captured using a magnifying equipment linked to a computerized camera.

3.13. Morphometric analysis

About 45 circular or roughly spherical seminiferous tubule sections were chosen from each group. The tubular diameter, germinal epithelium height, and lumen diameter was measured by using Image J software (Version 1.53i).

3.14. Comet assay testing for DNA damage analysis

The Single Cell Gel Electrophoresis (SCGE)/soluble comet measurement was done to observe single strand breakage in testicular tissue accordance with Nandhakumar [40]. To obtain a 10% tissue solution, samples were homogenized in cold homogenizer buffer (pH7.5) containing 75mM NaCl and 24mM Na2EDTA (pH13). A Closed-Circuit Digital (CCD) camera (Carl Zeiss Axioplan with epiflourescence utilizing channel 15 BP546/12, FT580, and LP590) inspected the prepared slides using a fluorescent magnifying lens at amplification 400X. Using Kinetic Imaging, Ltd. (Liverpool, UK) comet 5 picture examination algorithm, 50 cells for each specimen were employed to estimate DNA damage.

3.15. Statistical analysis

The current data were examined with the help of SPSS version 25, a statistical tool for social science software. To determine the differences between groups, analysis of Variance (ANOVA) was used, and followed by Tukey's multiple comparison post hoc analysis. Statistical significance was determined by using data expressed as mean ± standard error of the mean and a P-value of less than 0.05. Graph Prism was used for the analysis of Comet test.

4. Results

4.1. Weights of animal total body and reproductive organs

The body weight change diminished significantly in both treated groups (T1= 51.57±8.47 & T2= 27.714±4.87) compared to the control group (88.57±13.84). The absolute weight of the right cauda epididymis increased significantly in low dosage TMX group (0.30±0.02) compared to control group (0.19±0.01). The absolute weight of seminal vesicles, right and left testis and left cauda epididymis show non-significant distinction. However, TMX administration induced a significant elevation in relative weight of both testes (T1= 0.55±0.05 & 0.55±0.05 and T2= 0.65±0.09 & 0.63±0.09) as compared with the control group (0.53±0.08 & 0.53±0.06). While, caused non-significant increase in weight of cauda epididymis and seminal vesicles (Table1&2).

Table 1 displaying body weight change (g) and absolute weights of reproductive organs

Groups / Parameters	Control	Low (T_1) 156 mg/kg	High (T_2) 315 mg/kg
Seminal Vesicles	0.43±0.09	0.45±0.08	0.53±0.11
Left Testis	0.53±0.08	0.55±0.05	0.65±0.09[a]
Right Testis	0.50±0.06	0.55±0.04	0.63±0.09[a]
Left Cauda Epididymis	0.06±0.01	0.09±0.04	0.11±0.05
Right Cauda Epididymis	0.06±0.02	0.11±0.03	0.10±0.02

Each value represented as means ± SEM. a letter means there was a significant difference ($p < 0.05$) as compared with control group.

Table 2 displaying relative weights (%) of reproductive organs

Groups / Parameters	Control	Low (T_1) 156 mg/kg	High (T_2) 315 mg/kg
Body Weight Change/g	88.57±13.84	51.57±8.47[a]	27.71±4.87[a]
Seminal Vesicles Weight/g	1.20±0.11	1.16±0.09	1.277±0.08
Left Testis Weight/g	1.46±0.06	1.40±0.05	1.577±0.04
Right Testis Weight/g	1.40±0.059	1.41±0.05	1.537±0.01
Left Cauda Epididymis Weight/g	0.19±0.01	0.24±0.03	0.301±0.04
Right Cauda Epididymis Weight/g	0.19±0.01	0.30±0.02[a]	0.277±0.01

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group.

4.2. Effect of TMX on sperm quality

The sperm count, RRT, viability, motility, and progressive motility declined significantly in both TMX treated groups as compared to control group. TMX caused a significant decrease in the sperm count in both low dose group (22.36±0.40) and high dose group (20.48±0.29) when compared to the control group (28.29±0.69). The viability and motility percent decreased significantly in low treated group (50.0±3.65 & 60.83± 3.27) compared to the control group and (40.0±2.58 & 45.0±1.825) respectively. While, TMX exposure caused significant increase of fructose level at low TMX administration (46.72±4.16) and at high TMX administration (251.87±44.04) in comparison with control group (25.38±1.86). High TMX administration prompted significant drop of viability, motility, and progressive motility compared to the low treated group (36.57±3.57 & 50.0±3.65), (20.0±6.055 & 40.0±2.58) and (21.66±1.05 & 28.33±1.66) respectively (Table3).

Table 3 showing sperm quality outcome

Groups / Parameters	Control	Low (T_1) 156 mg/kg	High (T_2) 315 mg/kg
Fructose level	25.38±1.86	46.72±4.16[a]	251.87±44.04[a]
RRT	1.85±0.046	0.51±0.03[a]	0.42±0.05[a]
Viability (%)	60.83± 3.27	50.0±3.65[b]	36.57±36.57[a,b]
Motility (%)	45.0±1.825	40.0±2.58[b]	20.0±6.055[a,b]
Progressive Motility (%)	35.0±2.23	28.33±1.66[a,b]	21.66±1.05[a,b]
Sperm Count (No. of spermx10^6/MM)	28.29±0.69	22.36±0.400[a]	20.48±0.29[a]

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group. b letter means there was a significant difference between low & high treated groups.

4.3. Effect of TMX on sperm abnormalities

The administration of TMX alters spermatozoa morphology as shown in Figures (1-3). TMX significantly enhanced the bent tail, banana head, and pairing head incident. Low TMX dosage caused significant increase in number of sperm with head less abnormality (18.33±3.6) as compared with control (8.6±0.84). Also, high TMX dosage caused significant rise in number of sperm with bent neck aberration (81.5±16.74) as compared with control (4.33±0.33) and significant high level of pairing head as compared with low treated group (3.5±0.22 & 1.33±0.21). TMX administration showed a significant elevation in bent tail in both T1 group (49.0± 8.35) and T2 group (45.5±6.62) as compared with control (10.33± 3.18). Intake of TMX showed a significant increase in banana head in both low dose group (8.16±2.49) and high dose group (9.33±2.61) when compared to the control group (0.00±0.00). The incidence of total anomalies was reported, and imprecise form and conformation of either the head or the tail were regarded abnormal (Table 4).

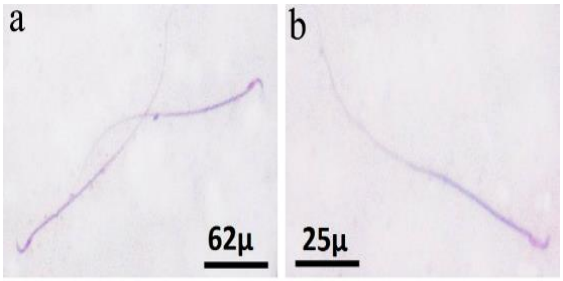

Fig. 1 Photographs showing normal appearance of spermatozoa from control group.

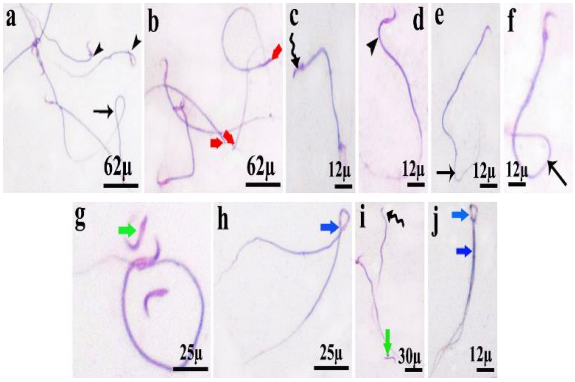

Fig. 2 Photographs showing sperm abnormalities from low dose group (T1). Bent neck (head arrow), bent tail (arrow), Headless sperms (red arrow), and Banana head without acrosome (wavy arrow), Tailless sperm (green arrow) and pairing phenomena (blue arrow).

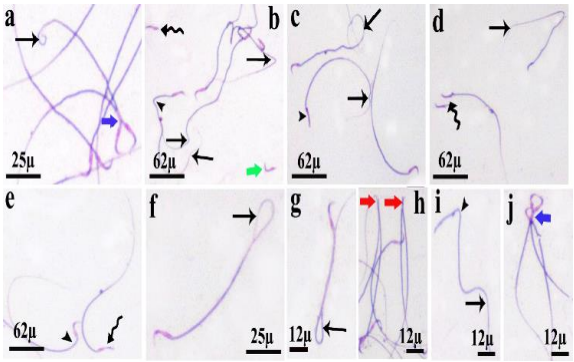

Fig. 3: Photographs showing sperm abnormalities from high dose group (T2). Bent neck (head arrow), bent tail (arrow), Headless sperms (red arrow), and Banana head without acrosome (wavy arrow), Tailless sperm (green arrow) and pairing phenomena (blue arrow).

Table 4 Presenting sperm morphological abnormalities

Group / Morphology %	Control	Low (T_1) 156 mg/ kg	High (T_2) 315 mg/ kg
Normal	298.5±23.8	104.66±4.08[a]	110.66±1.68[a]
Bent tail	10.33±3.18	49.0±8.35[a]	45.5±6.62[a]
Hook less	5.33±1.14	8.66±1.64	10±3.8
Head less	8.6±0.84	18.33±3.6[a]	14.5±1.97
Bent neck	4.33±0.33	44.33±10.43	81.5±16.74[a]
Tail less	16.66±0.802	40.16±4.94	41.16±9.48
Banana head	0±0	8.16±2.49[a]	9.33±2.61[a]
Pairing head	0±0	1.33±0.21[a,b]	3.5±0.22[a,b]

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group. b letter means there was a significant difference between low & high treated groups.

4.4. Effect of TMX on reproductive hormones

Testosterone levels diminished significantly in both treated groups as compared with the control group. High TMX administration induced significant increase in concentration of FSH (13.77±1.58) and LH (15.66±2.38) as compared with the control group (7.51±1.01 & 6.92±0.51) respectively. High treated group displayed a significant difference in the LH level compared to low treated group (15.66±2.38 & 9.35±1.22) respectively (Table 5).

Table 5 showing reproductive hormones level.

Groups / Parameters	Control	Low (T_1) 156 mg/ kg	High (T_2) 315 mg/ kg
Testosterone (ng/ml)	34.69±2.41	17.87±3.09[a]	17.97±0.00[a]
FSH (IU/L)	7.51±1.01	12.28±1.44	13.77± 1.58[a]
LH (IU/L)	6.92±0.51	9.35±1.22[b]	15.66±2.38[a,b]

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group. b letter means there was a significant difference between low & high treated groups.

4.5. Influence of TMX on testicular oxidative status

For oxidative stress parameters, the date showed clearly that the level of LPO, as indicated by MDA formation amplified significantly in high dose treated group (2764.35±714.47) compared to control group (1080.52±52.75). The glutathione (GSH) and catalase declined significantly in both T1 (4023.59±271.96 & 4905.91±150.76) (80.57±4.91 & 137.50±13.72) and T2 (3535.34±113.12 & 4905.91±150.76) as compared to control group (59.30±2.30 & 137.50±13.72) respectively (Table 6).

Table 6 showing level of testicular ROS markers.

Groups / Parameters	Control	Low (T_1) 156 mg/ kg	High (T_2) 315 mg/ kg
CAT(U/mg protein)	137.50± 13.72	80.57± 4.91[a]	59.30± 2.30[a]
GSH (U/g tissue)	4905.91± 150.76	4023.59± 271.96[a]	3535.34± 113.12[a]
MDA(nmol/g tissue)	1080.52± 52.75	1295.76± 22.24	2764.35± 714.47[a]

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group. b letter means there was a significant difference between low & high treated groups.

4.6. Testicular Histopathology examination

The testicular tissue from the control group showed the normal structure of the testicular parenchyma, which was made up of densely compacted seminiferous tubules and had the traditional characteristics of a normal structure. The spermatogenic cells in the seminiferous tubules were organized normally, with spermatogonia resting on the basement membrane, spermatocytes and spermatids, and spermatozoa within the lumen. Patches of Leydig interstitial cells were found in the interstitium (Fig. 4a). The testicular tissue of the treated groups exhibited disruption of seminiferous tubules with a noteworthy diminution in the spermatogenic cell series, as well as a significant number of seminiferous tubules lacking sperm, spermatids, and secondary spermatocytes. Within the lumen of some seminiferous tubules, exfoliation of injured spermatogenic cells was detected. The intertubular connective tissue showed a decrease in interstitial cells with oedema, enlargement of interstitial areas, vacuolation and extensive vascular thrombosis, as well as enlarged clogged vasculature. Most spermatogenic cells had pyknotic or uneven darkly pigmented nuclei and highly acidophilic cytoplasm, and some seminiferous tubules had inappropriate basement membranes (Fig. 4b-f).

4.7. Results of morphometric analysis

When compared to the control group (11.57±0.16) TMX exposure resulted in a substantial decrease in seminiferous tubule diameter (T1= 10.63±0.09) and (T2= 10.14±0.07). TMX intake caused a significant increase in epithelial germinal cell thickness (1.82±0.05) and (1.40±0.01) in comparison with control (2.56±0.06). TMX induced a significant increase in lumen diameter (5.47±0.12) and (6.43±0.09) as compared with control (4.13±0.07) (Table 7).

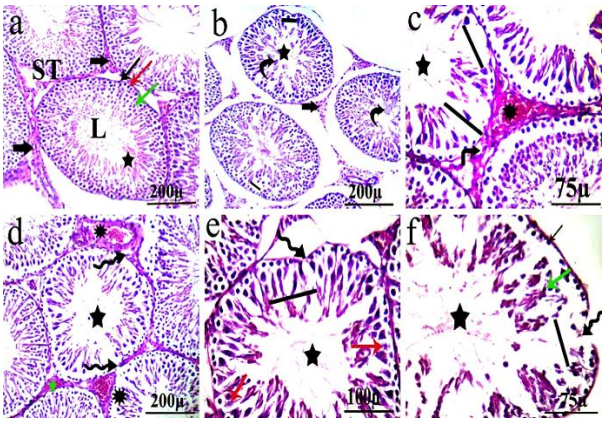

Fig. 4 Histopathological sections of testicular tissue. a: the testes sections from control group presented the normal viewing of testicular parenchyma consist of tightly packed seminiferous tubules (ST) and displayed the classical characters of usual structure. The seminiferous tubules showed normal organization of the spermatogenic cells, comprising spermatogonia (black arrow) rest on basement membrane, spermatocytes (red arrow) and spermatids (green arrow), and spermatozoa (star) inside lumen (L). The interstitium contained clusters of interstitial cells of Leydig (bold arrow). b-f: the testes sections from both treated groups showed disorganization of seminiferous tubules with noticeable reduction of the spermatogenic cell series (line). Number of the seminiferous tubules did not have sperm (star), spermatids (green arrow) and secondary spermatocytes (red arrow). Exfoliation of the damaged spermatogenic cells were found inside the lumen of some seminiferous tubules (turn arrow). The intertubular connective tissue revealed lessening of interstitial cells with markedly oedematous (green star), broadening of interstitial spaces (bold arrow), vacuolation (curved arrow) and massive vascular thrombosis and dilated congested blood vessels (asterisk). Some STs showed improper basement membranes (wavy arrow) and pyknotic or irregular darkly stained nuclei and deeply acidophilic cytoplasm were seen in most of spermatogenic cells.

Table 7 showing the morphometric scores

Groups / Parameters	Control	Low (T_1) 156 mg/ kg	High (T_2) 315 mg/ kg
Tubule diameter (µm)	11.57±0.16	10.63±0.09^{a,b}	10.14±0.07^{a,b}
Epithelial thickness (µm)	2.56±0.06	1.82±0.05^{a,b}	1.40±0.01^{a,b}
Lumen diameter (µm)	4.13±0.07	5.47±0.12^{a,b}	6.43±0.09^{a,b}

Each value represented as means ± SEM.
a letter means there was a significant difference ($p < 0.05$) as compared with control group. b letter means there was a significant difference between low & high treated groups.

4.8. DNA damage outcomes

TMX exposure resulted in DNA damage represented in a significant elevation in comet % (T1=17.46±1.30 & T2=22.60±1.35), tail length (T1=7.78±0.65 & T2= 9.56±0.16), and % DNA in tail (T1=10.61±0.10 & T2=10.67±1.02), tail moment (T1=0.93±0.08 & T2=1.22±0.11) and olive moment (T1= 1.00±0.01 & T2=1.1±0.06) was observed when compared to the control group (11.6±1.06 & 6.25±0.39 & 5.63±0.80 & 0.47±0.07 & 0.65±0.11) as shown in figures 5&6.

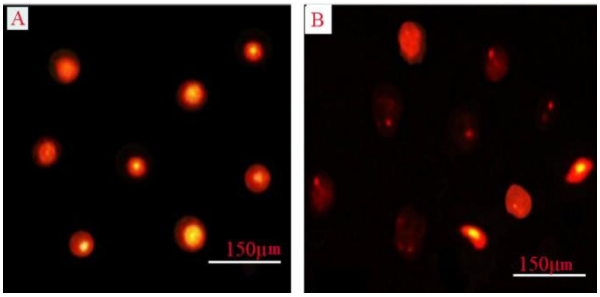

Fig. 5 Representative photos of testicular DNA damage induced by Thiamethoxam (B: Treated group compared with undamaged DNA (A: control group).

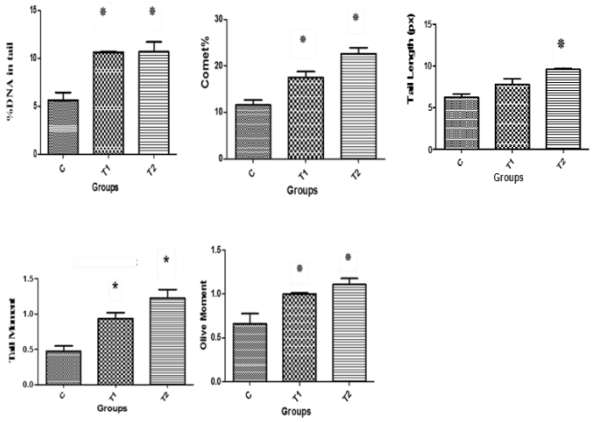

Fig. 6 DNA damage represented as % DNA in tail, Comet %, tail length, tail moment and olive moment in different treated groups in male testis. Results are expressed as mean ± SEM, (Asterisk) significant difference with control at $P < 0.05$.

5. Discussion

Recent studies reveal that neonicotinoid pesticides have detrimental reproductive toxicity consequences in animals, involving greater percentages of fetal loss, early delivery, lower conception rate, impaired sexual function, lower child weight, and miscarriage [41-42]. Thiamethoxam, a fairly recent pesticide, is a neonicotinoid pesticide from the second generation [43].

When comparing the TMX-treated rats to the control group, the weight gain change was significantly decreased in the TMX-treated rats. This could be due to inadequacy nutritional absorption caused by lower feed consumption and the immediate impacts of TMX on the gastrointestinal tract. These results are comparable to other scientists [44]. Furthermore, TMX administration resulted in a significant increase in the absolute and relative testicular weight when compared to control rats.

Administration of TMX at both dosage levels induced slight elevation of relative seminal vesicle and cauda epididymis weight. On the other hand, some pesticides were linked to lower sexual organ weights [45].

It is well established that measuring body weight, gives data on an organism's physical well-being, which could be useful in interpreting fertility effects [46]. The lower serum testosterone levels in the ongoing investigation could explain to the considerable declines in body mass index and the absolute weights of the right cauda epididymis and seminal vesicles [47].

Semen analysis is a useful tool for evaluating reproductive performance in human and animal models [48]. Throughout this research, TMX reduced semen quality by lowering sperm production, motility, and live/dead ratio, as well as boosting the number of morphologically aberrant spermatozoa in the TMX-treated rats compared to the control rats. Also, decreased the level of RRT and increased fructose level. The findings matched those of previous studies showing that imidacloprid (IMI) reduced the integrity of semen in experimental rats [49-51].

The number of sperm is significantly linked to abnormalities and histopathological consequences, both of which are dangerous, both of these are crucial markers of slightly earlier testicles damage induced by risky compounds exposure [52]. The significant decrease in seminal amount and quality observed in TMX-treated animals in the present study might be due to a lack of testosterone, which itself is required for proper sperm production [50,53]. Furthermore, the knowledge that TMX causes oxidative stress, which results in the generation of reactive oxygen species, might explain the lower semen quality [53]. Furthermore, because of the substantial polyunsaturated fatty acid content in spermatozoa's plasma membranes, they are especially sensitive to lipid peroxidation (LPO) [54]. As a result, LPO the most common symptom of oxidative stress induced by oxygen free radicals, can harm the lipid composition of sperm membranes, causing motion loss and membrane degradation, eventually contributing to sperm malfunctioning [55].

A reduction in spermatozoa motility, which seems linked to a minimal level of ATP concentration, can significantly limit reproductive capacity [56]. Furthermore, peroxidation of phospholipids, proteins, and nucleotides in the testicles resulted in a significant lessening in sperm motility and vitality, as well as a raise in spermatozoa deformities, as a result of an alteration in the proper functioning of multiple cytoplasmic organelles in the testis [57].

Resazurin reduction test (RRT) aid in the diagnosis of metabolically active sperms. RRT results appear to show the rate of reduction processes in spermatozoa instead of ATP generation in overall. Motile sperm create reducing equivalents (NAPH+H+ and NADH+H+) through metabolic processes. The existence of reducing nucleotides in spermatozoa indicates that they are active metabolically [58]. It has been hypothesized that resazurin is reduced to resorufin by diaphorases, which are NAD (P)-dependent enzymes [59].

Fructose provides energy to sperms. It is created by the seminal vesicles, with some help from the ductus deferens ampulla [60]. The measurement of seminal fructose content has been employed in the diagnosis of obstructive azoospermia and male accessory gland inflammation [61]. Assessment of seminal fructose as a biomarker of seminal vesicular integrity is supported by the World Organization handbook [62-63].

Because sperm utilize fructose as their main source of power, Gonzales (2001) found that a rise in sperm concentration is commonly associated with a drop in fructose content in seminal plasma [63-64]. As sperm concentration, vitality, motility, and morphology increase, greater energy is needed, hence fructose consumption decreases [65]. Adequate seminal fructose concentration verifies testosterone's role, and vesicle and vas deferens function are appropriate [63]. Fructose levels were discovered to be inversely related to sperm motility by [66], with $R = -0.062$ ($p<0.05$).

Testosterone is needed for sexual organ development, sperm formation process, and male fecundity [67], Long-term pesticide contamination disrupts hormones, causing reproductive problems [68]. It also lowers circulating hormonal levels by increasing steroid catabolism and removal [69]. The decline in sperm number reduction and sperm performance could be linked to a drop in blood testosterone levels as a result of TMX exposure, which is essential for optimal male reproductive organs development and function. This was supported by the significant decrease in testosterone levels in the serum of TMX-treated rats in the current investigation. These findings were consistent with previous research [70,49,50,51] proposed that a rise in testicles lipid peroxidation causes a decline in testis androgen formation, since elevated concentrations of corticosterone generated throughout oxidative stress could lead to a reduction in testosterone biogenesis as this steroid can stimulate Leydig cellular proliferation [56].

In the present study, TMX-treated rats had a considerable increase in blood LH and FSH levels and a significant decline in circulating testosterone levels when compared to the control group.

[57,71,47,51] obtained analogous outcomes in animals subjected to TMX, acetamiprid, and IMI. This means that TMX caused oxidants/antioxidant imbalance, which initiated the free radical creation and finally caused testicle cells damage that responsible to produce testosterone. Testicular steroidogenesis suppression could possibly play a role in the repression of testosterone production in Leydig cells [46]. FSH and LH that are released by the pituitary gland, control testosterone production. LH motivates Leydig cells to make testosterone, while FSH promotes Sertoli cells to discharge androgen-binding protein, which is prerequisite for maintaining proper testosterone level for germ cells during sperm production processes [72]. Testosterone, along with the gonadotropins FSH and LH, is the major hormone that controls spermatogenesis, sperm maturity, and sexual performance [73]. Low testosterone levels may produce negative response from the hypothalamus-pituitary-testicular (HPT) axis, subsequent greater serum LH and FSH levels [71].

CAT enzymes are necessary for the elimination of oxyradicals by neutralizing superoxide anion (O^{2-}) [74]. Furthermore, GSH is an essential antioxidative defense compound that aids in the detoxification process, participates as a co-substrate in antioxidant enzymatic responses accompanied by glutathione peroxidase and glutathione-S-transferase, and aids in the biodegradation of oxyradicals, as reported earlier after prolonged imidacloprid exposure [74,75]. The decrease of CAT activity found in our work suggests that TMX may considerably deplete the endogenous antioxidant status in testicle cells due to oxidative stress, as seen by the histopathology alterations identified in this research [75]. Also, the lower amounts of GSH found here could be attributable to its activity as an antioxidant in eliminating reactive oxygen species products and/or its use in the conjugation process [76].

MDA, additional hallmark of cell damage and ROS production, builds up as a result of lipid peroxidation and free radical damage to intracellular lipid components [76]. Since sperm mitochondrial membranes are high in polyunsaturated fatty acids and deficient in antioxidants, they are more susceptible to lipid peroxidation [46]. Due to the obvious devastating impact of ROS on lipid molecules, TMX exacerbated lipid peroxidation in male rats in the current investigation, which has been supported by our histopathological evaluation. In addition to creating reactive oxygen species (ROS) and generating oxidative stress [75].

Our findings revealed a significant rise in malondialdehyde (MDA) in high dose group when compared to the control group, as well as a significant reduction in glutathione (GSH) and catalase (CAT) in both treatment groups when compared to the control group. These findings indicated increased LPO and oxidative stress as a result of TMX treatment. This was in agreement with [77], who discovered that daily oral ingestion of Sulfx for 28 days at low (79.5 mg/kg) and high (205 mg/kg) doses resulted in significant increase in testicular MDA, GSSG, and NOx level versus a dose-dependent drop in GSH content. [51] discovered that giving imidacloprid (22.5 mg/kg b.wt) by orogastric each day for 56 days caused testicular free radical damage, as evidenced by exceptionally high malondialdehyde (MDA) levels, a noticeable downturn in antioxidant enzyme activity, and lower glutathione (GSH) and total antioxidant capacity (TAC) levels.

Dysfunctionality of seminiferous tubules with substantial decrease of the germ cells line, number of seminiferous tubules without sperm, widening of interstitial gaps, and certain Sertoli cells with inappropriate basement membranes were observed in the testicles of rats treated with TMX. In addition, our results showed a significant reduction in tubule diameter, epithelial thickness, and lumen diameter in both treated groups. This view is supported by [78], who found adolescent histological morphology of the testicle in males treated with TMX, as evidenced by dramatic declines in spermatogenesis, reasonable tubular atrophy, and higher occurrences of spermatic giant cells. This was in line with the findings of [77], who discovered that Sulfx administration, particularly at high doses, resulted in a testis damage characterized by a decrease in testicles morphometric parameters such as the diameter and size of seminiferous tubules due to germ cell spermatogenic deterioration. In addition, [70] discovered that treating male Wistar rats with imidacloprid (45 and 90 mg/kg, b.wt) for 28 days caused oxidative stress, which resulted in histopathological deficient spermatogenesis, as evidenced by spermatocyte exhaustion and testis and epididymal deteriorating alterations.

Furthermore, oxidative stress caused by Clothianidin at a dose of 50 mg/kg body weight accelerated DNA fragmentation in the seminiferous tubules, elevated vacuolization in the seminiferous epithelial and reduced germ cells numbers in mature male quails in a dose-dependent manner [79].

In the proposed investigation, testicles feature alteration was linked to structural injuries such as fewer spermatogenesis in certain tubules, the existence of either excess intratubular vacuolations or exfoliated necrotic spermatogenic cells, as well as spermatogenic cell deformation, deterioration, intertubular edoema. These results supported those of [80], who found that high dosages of TMX had testicles effects in a multi-generation reproduction research [57,53].

In comparison to the control group, the current findings revealed considerable DNA damage in the treatment groups in male testis as measured by percent DNA in tail, Comet percent, tail length, tail moment, and olive moment. This was in line with the findings of [77], who discovered that Sulfx treatment caused a significant increase in testicular DNA fragmentation, indicating poor spermatogenesis [53]. On the other hand, found that TMX administration did not result in considerable sperm DNA fragmentation. The large amount of poly unsaturated fatty acids (PUFAs) in mammalian spermatozoa could explain this. As a result, ROS can target the unsaturated bonds of lipids in the sperm membrane and disturb the architecture of the lipid matrix in spermatozoa membranes, causing different deformities [81,82]. Oxidative stress has been proven to degrade sperm phospholipid, DNA, and proteins, as well as promote apoptotic cell death, resulting in sperm quality reduction and sperm performance degradation [83,76].

Finally, the current study demonstrates that TMX exposure resulted in a drop in sperm quality, concentration, disintegration of testicles DNA, and antioxidants insufficiency. All of the above alterations point to a reduction in testicular function, and reveal a detrimental impact of TMX on male rats' reproductive performance. As a consequence, the present experiment's findings imply that TMX administration caused serious damage, presumably by generation of oxidative stress, which could lead to testicles malfunction. As a result, we should be informed that TMX exposure may be hazardous to the reproductive organs, and essential care should be implemented to reduce the deleterious impacts of TMX on man and animal species in order to counteract contamination.

Table. Represented all used terminologies with abbreviations

Terminology	Abbreviation
Agriculture Research Centre	ARC
Catalase	CAT
Deoxyribonucleic Acid	DNA
European Society for Human Reproduction and Embryology	ESHRE
Follicle-Stimulating Hormone	FSH
Glutathione peroxidase	GSH
Haematoxylin and Eosin	H&E
High-Affinity Choline Uptake	HACU
Luteinizing Hormone	LH
Malondialdehyde	MDA
Polyunsaturated Fatty Acids	PUFAs
Reactive Oxygen Species	ROS
Resazurin Reduction	RRT
Single Cell Gel Electrophoresis	SCGE
Thiamethoxam	TMX

6. Conflict of Interest

The authors declare no conflict of interest.

7. Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

8. Acknowledgements

We lively to all people support us to achieve this work at Zoology Department, Al-Azhar and Cairo Universities.

9. Author contributions

All authors contributed similarly.

10. References

[1] Wangnoo K.S., Evaluation of Male Infertility. J Int Med Sc Acad, 23(2) 107-9(2010).

[2]World Health Organization (WHO), Laboratory manual for the examination and processing of human semen, Cambridge University Press, Cambridge, United Kingdom, 5th Edn. (2017).

[3] Schafer K., Pesticides and male infertility: harm from the womb through adulthood and into the next generation. Environmental Health Policy Institute (PSR),(2010).

[4] Sharma A.D., Mallick A.R., Ghosh A.K., Free radicals and their role in different clinical conditions: An overview. Int J Pharm Sci Res, 1, 185-192(2010). https://bit.ly/31BZYFQ.

[5] Kimura-Kuroda, J., Komuta Y., Kuroda Y., Hayashi M., Kawano H., Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. PLoS One, 7, e32432(2012).

[6] Lundin O., Rundlöf M., Smith H.G., Fries I., Bommarco R., Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps, PloS one,10, e0136928(2015).

[7] Jeschke P., Nauen R., Neonicotinoids from zero to hero in insecticide chemistry. J Pest Manag Sci, 64, 1084–1098 (2008).

[8] Straub L., Villamar-Bouza L., Bruckner S., Chantawannakul P., et al., Neonicotinoid insecticides can serve as inadvertent insect contraceptives, In Proc. R. Soc. B., 283(1835), 20160506(2016).

[9] Hoshi N., Hirano T., Omotehara T., Tokumoto J., Umemura Y., Mantani Y., Tanida T., Warita K., Tabuchi Y., Yokoyama T., Kitagawa H., Insight into the mechanism of reproductive dysfunction caused by neonicotinoid pesticides. J Biol Pharm Bull, 37, 1439-1443(2014).

[10] Kimura-Kuroda J., Komuta Y., Kuroda Y., Hayashi M., Kawano H., Nicotine-like effects of the

neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. J PloS One, 7, e32432-e32443(2012).
[11] Bal R., Naziroğlu M., Türk G., Ökkes Y., Kuloğlu T., Etem E., Baydas G., Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. J Cell Biochem Funct, 30, 492-499 (2012).
[12] Lin P.C., Lin H.J., Liao Y.Y., Guo H.R., Chen K.T., Acute poisoning with neonicotinoid insecticides: a case report and literature review. J Basic Clin Pharmacol Toxicol, 112, 282-286 (2013).
[13] Goulson D., REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides. J Appl Ecol, 50, 977-987(2013).
[14] Maienfisch P., Angst M., Brandl F., Fischer W., Hofer D., Kayser H. et al., Chemistry and Biology of Thiamethoxam: a Second Generation Neonicotinoid. Pest Management Science, 57(10),906-913 (2001).
[15] Sams C., Cocker J., Lennard M.S., Biotransformation of chlorpyrifos and diazinon by human liver microsomes and recombinant human cytochrome P450s. Xenobiotica, 34(10),861-873 (2004).
[16] Elhamalawy, O. H., Al-Anany, F. S., & El Makawy, A. I., Thiamethoxam-induced hematological, biochemical, and genetic alterations and the ameliorated effect of Moringa oleifera in male mice. Toxicology Reports (2022).
[17] Green T., Toghill A., Lee R., Waechter F., Weber E., Peffer R., Robinson M., Thiamethoxam induced mouse liver tumors and their relevance to humans: Part 2: Species differences in response. Toxicological Sciences, 86(1), 48-55(2005).
[18] Arfat Y., Mahmood N., Tahir M.U., Rashid. M., Anjum S., Zhao F., Qian A. R., Effect of imidacloprid on hepatotoxicity and nephrotoxicity in male albino mice. Toxicology reports, 1, 554-561(2014).
[19] Rodrigues K.J.A., Santana M.B., Do-Nascimento J.L.M., Behavioral and biochemical effects of neonicotinoid thiamethoxam on the cholinergic system in rats. Ecotoxicology and Environmental Safety, 73 (1), 101-107(2010).
[20] Shalaby S.E.M., Farrag A.H., El-Saed G.S.M., Toxicological potential of thiamethoxam insecticide on albino rats and its residues in some organs. Journal Arab Society Medical Research 5 (2), 165-172 (2010).
[21] Shadnia S., Moghaddam H.H., Fatal intoxication with imidacloprid insecticide. American Journal of Emergency Medicine, 26(5):634 (2008).
[22] Bretveld R., Brouwers M., Ebisch I., Roeleveld N., Influence of pesticides on male fertility. Scandinavian Journal Work Environment and Health. 33(1),13- 28 (2007).
[23] Pina-Guzman B., Solis-Heredia M.J., Quintanilla-Vega B., Diazinon alters sperm chromatin structure in mice by phosphorylating nuclear protamines. Toxicology and Applied Pharmacology Journal. 15,189-198(2005).
[24] Altuntas I., Kilinc I., Orhan H., Demirel R., Koylu H., Delibas N., The effects of diazinon on lipid peroxidation and antioxidant enzymes in erythrocytes in vitro. Human and Experimental Toxicology Journal, 23,9-13(2004).
[25] Maxwell L.B., Dutta H.M., Diazinon-induced endocrine disruption in bluegill sunfish, Lepomis macrochirus. Ecotoxicology and environmental safety, 60(1), 21-27(2005).
[26] Rose P.H., Nicotine and the neonicotinoids, In:T.C.Marrs editor. Mammalian Toxicology of Insecticides. Royal Society of Chemistry, Cambridge, 184(2012).
[27] Swenson T.L., Casida J.E., Neonicotinoid formaldehyde generators: possible mechanism of mouse-specific hepatotoxicity/hepatocarcinogenicity of thiamethoxam. Toxicology Letter, 216 (2-3) 139-145(2013).
[28] Creasy D.M., Evaluation of testicular toxicity in safety evaluation studies: The - appropriate use of spermatogenic staging. Toxicol Pathol, 25, 119-131(1997).
[29] Morrisey R.E., Schwetz B.A., James C., Monica D.R., Teague J.L., Moris R.W., Evaluation of rodent sperm, vaginal cytology and reproductive organ weight from NationalToxicology Programme — 13-week studies. Fundam. Appl. Toxicol, 11, 343–358(1988).
[30] Freund M., Carol B., Factors affecting haemocytometer counts of sperm concentration in human semen. Reproduction, 8(2), 149-155(1964).
[31] Pant N., Srivastava S.P., Testicular and spermatotoxic effect of quinaphos in rats. J. Appl. Toxicol, 23, 271-274(2003).
[32] Reddy K., Bordekar A.D., Spectrophotometric analysis of resazurin reduction test and semen quality in men. Indian J. Exp. Biol, 37 (8), 782 – 786(1999).
[33] Foreman D., Gaylor L., Evans E., Trella C., A modification of the Roe procedure for determination of fructose in tissues with increased specificity. Analytical biochemistry, 56(2), 584-590(1973).
[34] Blom E., A one minute live-dead sperm stain by means of eosin-nigrosin. Journal of Fertility and Sterility, 1, 176-177(1950).
[35] Morakinyo A.O., Iranloye B.O., Adegoke O.A., Antireproductive effect of calcium channel blockers on male rats. Reprod. Med. Biol, 8, 97-102(2009).
[36] Satoh K., Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method,Clinica Chimica Acta, 90, 37-43(1978).
[37] Beutler E., Olga D., Barbara M.R., Improved method for the determination of blood

glutathione.Journal of Laboratory and Clinical Medicine, 6(1), 882-888(1963).
[38] Aebi H., 13 Catalase in vitro. Methods in enzymology, 105, 121-126(1984).
[39] Bancroft C.F., Gamble M., 5th ed. Churchill Livingstone Edinburgh, London (2002).
[40] Nandhakumar S., Parasuraman S., Shanmugam M.M., Rao K.R., Chand P., Bhat B.V., Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). Journal of pharmacology & pharmacotherapeutics, 2(2), 107(2011).
[41] Gibbons D., Morrissey C., Mineau P., A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environmental Science and Pollution Research, 22(1), 103-118(2015).
[42] Berheim E. H., Jenks J.A., Lundgren J.G., Michel E.S., Grove D., Jensen W.F., Effects of neonicotinoid insecticides on physiology and reproductive characteristics of captive female and fawn white-tailed deer. Scientific reports , 9(1), 1-10(2019).
[43]Thompson D.A., Lehmler H.J., Kolpin D.W., Hladik M.L., Vargo J.D., Schilling K.E., ... & Field RW. A critical review on the potential impacts of neonicotinoid insecticide use: current knowledge of environmental fate, toxicity, and implications for human health. Environmental Science: Processes & Impacts, 22(6), 1315-1346(2020).
[44] Hassan A.M.S., Abo El-Ela F.I., Abdel-Aziz A.M.H., Investigating the potential protective effects of natural product quercetin against imidacloprid-induced biochemical toxicity and DNA damage in adults rats. Toxicol. Rep, 6, 727–735(2019).
[45] Mallick C., Mandal S., Barik B., Bhattacharya A., Ghosh D., Protection of testicular dysfunctions by MTEC, a formulated herbal drug, in streptozotocin induced diabetic rat. Biol Pharm Bull, 30, 84–90(2007).
[46] Bal R., Türk G., Yılmaz Ö., Etem E., Kuloğlu T., Baydaş G., Naziroğlu M., Effects of clothianidin exposure on sperm quality, testicular apoptosis and fatty acid composition in developing male rats. Cell biology and toxicology, 28(3), 187-200(2012).
[47] Arıcan E.Y., Kayalı D.G., Karaca B.U., Boran T., Öztürk N., Okyar A., Özhan G., Reproductive effects of subchronic exposure to acetamiprid in male rats. Scientific reports, 10(1), 1-10(2020).
[48] Giribabu N., Reddy P.S., Protection of male reproductive toxicity in rats exposed to di-n-butyl phthalate during embryonic development by testosterone. Biomedicine & pharmacotherapy, 87, 355-365(2017).
[49] Mohamed A.A., Mohamed W.A., Khater S.I., Imidacloprid induces various toxicological effects related to the expression of 3β-HSD, NR5A1, and OGG1 genes in mature and immature rats. Environmental Pollution, 221, 15-25(2017).
[50] Tetsatsi A. C., Nkeng-Effouet P. A., Alumeti D.M., Bonsou G.R., Kamanyi A., Watcho P., Colibri® insecticide induces male reproductive toxicity: alleviating effects of Lannea acida (Anacardiaceae) in rats. Basic and clinical andrology, 29(1), 1-13(2019).
[51] Saber T.M., Arisha A.H., Abo-Elmaaty A.M., Abdelgawad F.E., Metwally M.M, SaberT., Mansour M.F., Thymol alleviates imidacloprid-induced testicular toxicity by modulating oxidative stress and expression of steroidogenesis and apoptosis-related genes in adult male rats. Ecotoxicology and Environmental Safety, 221, 112435(2021).
[52] McGregor M.B., Roland S., Thiamethoxa Toxicity Evaluation Consultants, Aberdour, Scotland Federal Institute for Risk Assessment, Berlin, Germany, 611-623(2010).
[53] El-Sawasany A.R., El Okle O.S., El Euony O.I., Nazem A.M., Impact of the Neonicotinoid Insecticide, Thiamethoxam, on Some Reproductive Parameters in Adult Male Rabbits. Alexandria Journal of Veterinary Sciences, 51(2), 386-392(2016).
[54] Alvarez J. G., Storey B.T., Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Molecular reproduction and development, 42(3), 334-346(1995).
[55] Adedara I.A., Owoeye O., Awogbindin I.O., Ajayi B.O., Rocha J.B., Farombi E.O., Diphenyl diselenide abrogates brain oxidative injury and neurobehavioural deficits associated with pesticide chlorpyrifos exposure in rats. Chemico-biological interactions, 296, 105-116(2018).
[56] Bal R., Türk G., Tuzcu M., Yilmaz O., Kuloglu T., Gundogdu R., Etem E., Assessment of imidacloprid toxicity on reproductive organ system of adult male rats. Journal of Environmental Science and Health, Part B, 47(5), 434-444(2014).
[57] El-Okle O.S., Lebda M.A., Tohamy H.G., Thiamethoxam-induced biochemical, hormonal and histological alterations in rats. Int. J. Toxicol. Pharmacol. Res, 8, 320-325(2016).
[58] Zalata A.A., Lammertijn N., Christophe A., Comhaire F.H., The correlates and alleged biochemical background of the resazurin reduction test in semen. International journal of andrology, 21(5), 289-294(1998).
[59] Strzeżek R., Filipowicz K., Stańczak M., Kordan W., Spectrophotometric analysis of the resazurin reduction test as a tool for assessing canine semen quality. Bull. Vet. Inst. Pulawy, 57, 281-285(2013).
[60] Schoenfeld C.Y., Amelar R.D., Dubin L., Numeroff M., Prolactin, fructose, and zinc levels

found in human seminal plasma. Fertility and sterility, 32(2), 206-208(1979).
[61] Manivannan B., Bhande S.S., Panneerdoss S., Sriram S., Lohiya N.K., Safety evaluation of long-term vas occlusion with styrene maleic anhydride and its non-invasive reversal on accessory reproductive organs in langurs. Asian journal of andrology, 7(2), 195-204(2005).
[62] Franken D.R., Oehninger S., Semen analysis and sperm function testing. Asian journal of andrology,14(1), 6(2012).
[63] Trang N.T., Huyen V.T., Linh N.T., Sang T.T., Seminal fructose concentration in man infertility and the fructose test's meaning in diagnosis reason of azoospermia man. Biomed J Sci & Tech Res, 8(1), 6270-6274(2018).
[64] Gonzales G.F., Function of seminal vesicles and their role on male fertility. Asian journal of Andrology, 3(4), 251-258(2001).
[65] Lu J.C., Chen F., Xu H.R., Huang Y.F., Lu N.Q., Standardization and quality control for determination of fructose in seminal plasma. Journal of andrology, 28(2), 207-213(2007).
[66] Buckett W.M., Lewis-Jones D.I., Fructose concentrations in seminal plasma from men with nonobstructive azoospermia. Archives of andrology, 48(1), 23-27(2002).
[67] Pidoux G., Gerbaud P., Tsatsaris V., Marpeau O., Ferreira F., Meduri G., Frendo J.L., Biochemical characterization and modulation of LH/CG—receptor during human trophoblast differentiation. Journal of cellular physiology, 212(1), 26-35(2007).
[68] Cardone A., Imidacloprid induces morphological and molecular damages on testis of lizard (Podarcis sicula). Ecotoxicology , 24(1), 94-105(2015).
[69] Civen M., Brown C.B., The effect of organophosphate insecticides on adrenal corticosterone formation. Pesticide Biochemistry and Physiology, 4(3), 254-259(1974).
[70] Lonare M., Kumar M., Raut S., More A., Doltade S., Badgujar P., Telang A., Evaluation of ameliorative effect of curcumin on imidacloprid-induced male reproductive toxicity in wistar rats. Environmental toxicology , 31(10), 1250-1263(2016).
[71]Kong D., Zhang J., Hou X., Zhang S., Tan J., Chen Y., Cai R., Acetamiprid inhibits testosterone synthesis by affecting the mitochondrial function and cytoplasmic adenosine triphosphate production in rat Leydig cells. Biology of reproduction, 96(1), 254-265(2017).
[72] Kackar R., Srivastava M.K., Raizada R.B., Assessment of toxicological effects of mancozeb in male rats after chronic exposure. NISCAIR-CSIR, India, 37(6),553-559, (1999).
[73] Ali R.I., Ibrahim M.A., Malathion induced testicular toxicity and oxidative damage in male mice: the protective effect of curcumin. Egyptian Journal of Forensic Sciences , 8(1), 1-13(2018).
[74] Al-Megrin W.A., Alkhuriji A.F., Yousef A.O., Metwally D.M., Habotta O.A., Kassab R.B., El-Khadragy M.F., Antagonistic efficacy of luteolin against lead acetate exposure-associated with hepatotoxicity is mediated via antioxidant, anti-inflammatory, and anti-apoptotic activities. Antioxidants, 9(1), 10(2020).
[75] Habotta O.A., Ateya A., Saleh R.M., El-Ashry E.S., Thiamethoxam-induced oxidative stress, lipid peroxidation, and disturbance of steroidogenic genes in male rats: Palliative role of Saussurea lappa and Silybum marianum. Environmental Toxicology, 36(10), 2051-2061(2021).
[76] Albarakati A.J., Baty R.S., Aljoudi A.M., Habotta O.A., Elmahallawy E.K., Kassab R.B., Moneim A.E., Luteolin protects against lead acetate-induced nephrotoxicity through antioxidant, anti-inflammatory, anti-apoptotic, and Nrf2/HO-1 signaling pathways. Molecular biology reports, 47(4), 2591-2603(2020).
[77] Said N.I., Abd-Elrazek A.M., El-dash H.A., The protective role of resveratrol against sulfoxaflor-induced toxicity in testis of adult male rats. Environmental Toxicology, 36(10), 2105-2115(2021).
[78] Dutta H.M., Meijer H.J., Sublethal effects of diazinon on the structure of the testis of bluegill, Lepomis macrochirus: a microscopic analysis. Environmental Pollution, 125(3),355-360(2003).
[79]Tokumoto J., Danjo M., Kobayashi Y., Kinoshita K., Omotehara T, Tatsumi A., Hashiguchi M., Sekijima T., Kamisoyama H., Yokoyama T., Kitagawa H., Hoshi N., Effect of exposure to clothianidin on the reproductive system of male quails. Journal of Veterinary Medical Science, 75(6), 755-760(2013).
[80] Breckenridge C.B. and Stevens J.T., "Crop protection chemicals: mechanism of action and hazard profiles,"In: A.W.Hayes,editor. Principles and methods of toxicology",5th Edition, Editado por A. Wallace Hayes, (2008).
[81] Aitken R. J., Roman S.D., Antioxidant systems and oxidative stress in the testes, Adv. Exp. Med. Biol. 636,154-171(2008). https://doi.org/10. 1007/978-0-387-09597-49.
[82] Kothari S., Thompson A., Agarwal A., du Plessis S.S., Free radicals: their beneficial and detrimental effects on sperm function. Indian.J.Exp.Biol, 48,425-435(2010).
[83] Walczak-Jedrzejowska R., Wolski J.K., Slowikowska-Hilczer J., The role of oxidative stress and antioxidants in male fertility. Cent European J Urol, 66(1), 60(2013).