Toxic impacts of a mixture of three pesticides on the reproduction and oxidative stress in male rats

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Abstract The present study investigated the toxic effect of a mixture of three pesticides (cypermethrin, mancozeb, and metalaxyl) on reproduction and oxidative stress parameters in male *Wistar* rats. Animals were treated at doses 1/60, 1/30, and 1/10 LD50 of each pesticide daily in the diet for 08 weeks. At the end of the treatment period, animals were sacrificed by decapitation. The results indicate a decrease in the absolute weight of testes and epididymis, the serum of testosterone hormone, and cholesterol levels. These parameters were significant reduced in males exposed to the mixed pesticides. A reduction in sperm concentration, motility, and viability also was observed. Besides, the ingestion of mixed pesticides at all three concentrations caused a significant decrease in GSH, GPx levels and an increase in MDA levels compared to the control group. This was accompanied by histopathological changes in testis and epididymis of rats such as seminiferous tubules degeneration, decreasing number of spermatogenic cells, edema, expansion of interstitial spaces, cell necrosis, and reducing the diameter of the epididymal tube compared to the control group. Thus, we strongly suggest that the mixture of pesticides causes damages to the male reproductive system.

Keywords: cholesterol, histopathology of testes and epididymis, MDA, testosterone

1. Introduction

Pesticide plays an important role in agricultural space or public health protection programs for preventing and controlling plants from pests and vector-borne diseases, leading to increased food production (Alewu and Nosire 2011). Although, the over-application or misuse of these pesticides caused a possible adverse effect on human and animal health (Balali-Mood 2008).

The population is exposed either simultaneously or sequentially to more than one pesticide every day via multiple pathways (contaminated food and water, household insecticides application). However, those routes are less dangerous to human life than the groups exposed daily for many hours, such as producers, pesticide workers, and farmers (Aktar et al 2009). The frequent use of pesticides in agricultural and commercial settings has led some researchers to study the effects of mixtures of these compounds as they co-occur in the environment (Trimble and Lydy 2006). Many studies demonstrated that exposure to pesticides might contribute to various diseases, including neurological, carcinogenic (Bassil et al 2007), respiratory (Hernández et al 2011), reproductive and developmental toxicity (Hanke and Jurewicz, 2004)**.** Furthermore, many studies have shown that high occupational to pesticides may provoke oxidative stress damages to various physiological function systems as testicular function. (Ihsan et al 2011; Mehrpour et al 2014).

This study tested three chemical pesticides, an insecticide (Cypermthrin) and two fungicides (mancozeb and metalaxyl), which are all widely used in agriculture in the east of Algeria to control insect and fungal diseases in a variety of corps (Sukul and Spiteller 2000; Joshi et al 2005; Solat et al 2010). Many studies have investigated that cypermethrin and metalaxyl altered the nervous system and induced histochemical damage in liver tissues (Okdah 2005; Noaishi et al 2013). Likewise, mancozeb exposure can affect the function of the thyroid gland by decreasing serum thyroxine levels, thyroid peroxidase (TPO) activity, and iodine uptake (Goldner et al 2010). Moreover, they can induce histopathological changes in the liver of animals, such as vacuolation of hepatocytes, Necrosis, and infiltration of inflammatory cells (Elkhansa et al 2015).

The reproductive system is a sensitive structure that can be affected upon exposure to toxic compounds (Shakkebaek 2001). Male infertility is estimated at 40% of infertility cases (Sadock et al 2003), and it may be linked to exposure to pesticides (Sharma et al 2014). Recent studies have shown that cypermethrin, mancozeb, and metalaxyl affect spermatogenesis, reducing semen quality of the testes and epididymis in exposed males (Main et al 2002; Sunder 2002; Joshi et al 2005). Other researchers have found that pesticides could also disturb the hypothalamic-pituitary-gonadal axis (Alaa-Eldin et al 2016). On the other hand, these toxicants can produce oxidative stress in reproductive tissues

by forming free radicals (ROS), leading to cellular apoptosis in testis and epididymis and perturbation of antioxidant profile defenses.(Diazveliz 2004; Joshi et al 2010) Besides, excessive production of ROS can impair reproductive capacities by modifying sperm mobility, viability, and integrity of their membrane (Abd-Ellah et al 2015; Gririchi et al 2017). Thus, the toxicity potential of these pesticides has been extensively studied, and a great deal of data has been collected. However, about 95% of these studies have been conducted on individual compounds (Groten 2001). Although currently, the majority of pesticides are used in combination. The local manufacturers are formulating mixtures of ready-to-use pesticides (British 2013) instead of using individual ones, which may cause more health damage. The potential effects of these mixtures on the reproductive system remain unknown and poorly understood (Wade et al 2002).

Therefore, this research aimed to evaluate the adverse effects of the three pesticides mixture (cypermethrin, mancozeb, and metalaxyl) on the reproductive system and oxidative stress in male Wistar rats.

2. Materials and Methods

2.1. Animals and treatment

Twenty-seven male Wistar rats weighing between 200 to 250 g were obtained from the Animal Center of Alger in Algeria. The rats were acclimatized for two weeks, and the diet and water were given *ad libitum*. Animals were maintained in a good rearing condition of ambient temperature and a light-dark photoperiod. After the acclimation period, the animals were divided into four groups with eight rats each. Animals were treated with the mixed pesticide 1/60, 1/30, and 1/10 LD50 of the used pesticides (Cypermethrin, Mancozeb, and Metalaxyl) daily in the diet for 08 weeks. At the end of treatment, animals were sacrificed by decapitation, blood and semen were collected. Serum was collected for hormone and cholesterol assays. The right testes and epididymis were excised and weighed. The right testis was frozen for sperm production and determination of oxidative stress parameters. The left one was processed for histopathology. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba, before starting the experimental work.

2.2. Semen analysis

Semen parameters (concentration and motility) were measured using Computer Assisted Semen Analysis (CASA). After decapitation, the semen was collected from a small opening in the epididymis cauda. About 1 µl of semen was diluted in 0.09% physiological solution; after that, about 5 µL of the mixture was placed in the Goldcyto counting chamber using a micropipette. This preparation was examined under a microscope connected to a microcomputer at a final magnification of x40.

2.3. Hypo-osmotic swelling (HOS) test

The HOST test was used to evaluate the integrity of sperm by exposing a drop of sperm, derived from the epididymis cauda, to the hypo-osmotic solution composed of fructose and sodium citrate (Jeyendran 1984), after which 100 spermatozoa were observed. The number of live sperms (as shown by an inflation of the tail) were counted.

2.4. Testosterone and cholesterol levels

Testosterone levels were determined in serum rats by immunoassay analyzer Access 2. Cholesterol levels were estimated by the clinical chemistry analyzer (MINDRAY BS-240), using commercial kits and colorimetric enzymatic method.

2.5. Histology examination

The organs were fixed in formol 10% and dehydrated in a series of [70–100%] ethanol baths. Then, they were placed in paraffin at 58°C for inclusion. Sections of 4 to 6µm were prepared from paraffin blocks using a rotary microtome and then were stained with Hematoxylin-Eosin (H- E) using Martoja and Martoja (1967) method. All sections were photographed with a specific camera in a Leica microscope.

2.6. Measurement of oxidative stress parameters

Malondialdehyde (MDA) was measured according to the method described by Ohkawa et al (1979). Total glutathione (GSH) content in blood was evaluated by the method detailed by Weckbecker and Cory (1988). The Glutathione peroxidase activity was measured spectrophotometrically by the method of Flohé and Günzler (1984). The protein concentration was determined according to the Bradford method (1976).

2.7. Statistical analysis

Results were expressed as mean±SD. The statistical analysis of the data was performed by the test of *t*-Student pairs between the control group and each treated group. The statistical analysis was carried out by using the Graph Pad Prism software (version 5). Significant differences was considered when $*P < 0.1$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1. The effect of the pesticides mixture on the testis and epididymis absolute weight

There was no significant decrease in the weight of testis and epididymis in group G2. However, we noticed a significant reduction ($P < 0.01$, $P < 0.01$) in groups treated with the medium (G3) and high doses of the mixture (G4) compared to the control group G1 (Figure 1).

3.2. Effect of the mixture on sperm count, motility, viability

It was remarked in (Table 1), a non-significant reduction in sperm count and motility in the treated group G2 compared to the control group G1. However, we have

found a higher and very higher significant ($P < 0.01$, $P < 0.001$) reduction in groups G3 and G4, respectively, compared to the control G1.

The non-significant influence of mixture pesticide in the normal sperm in group G2 compared to the control (Table 1), while in group G3 and G4, we observed a significant increase ($P < 0.05$, $P < 0.01$) to compare to the control group. The malformation of the tail of sperm type (A, B, and C) increased in groups G3 and G4, but group G2 non significantly affected ($P > 0.05$) compared to the control animals.
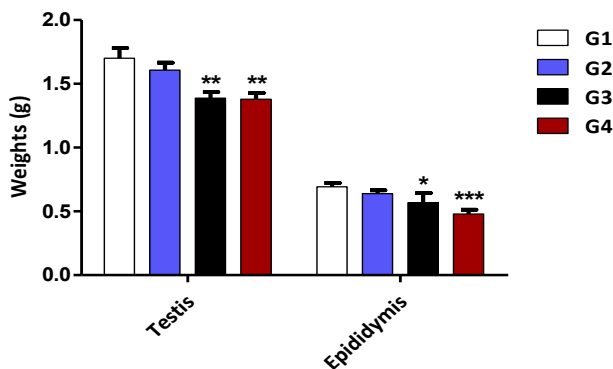

Figure 1 Effect of the mixture of pesticides on rats' testicular and epididymis weights (MV±SD, n = 9). **$P < 0.01$; ***$P < 0.001$.

3.3. Effect of the mixture on serum testosterone and cholesterol level

The change in testosterone level in all groups of rats is illustrated in figure 2. The results show a non-significant change in serum testosterone levels of treated animals in group G2 compared to the control group G1. On the other hand, a significant reduction ($P < 0.05$, $P < 0.01$) was deduced in groups G2 and G3 compared to the control group.
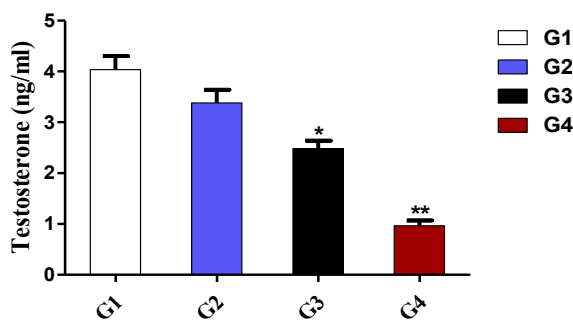

Figure 2 Evaluation of testosterone level (ng/ml) of rats exposed to mixture pesticide after eight weeks of treatment. Results are expressed as (Mean±SD, n=8). * $P < 0.1$; ** $P < 0.01$.

The results in figure 3 show a non-significant change in serum cholesterol levels of treated animals in group G2 compared to the control group G1. While, a higher significant increase ($P < 0.05$, $P < 0.01$) was deduced in group G2 and G3 compared to the control group G1.

The control group (AA') showed normal spermatogenesis cells in different phases and the typical cell arrangement in the somniferous tubules. However, we have observed a reduction in spermatozoid cells in the lumen in the treated group (B B'). Also in the treated groups (CC' and DD') showed degeneration in the seminiferous tubule. The interstitial spaces are enlarged due to tubular atrophy, edema, and a decrease in the number of sperms in the lumen.

The control groups (AA') showed the ducts' regular and normal external counter into the epididymal caput. Although, the treated groups (BB', CC' and DD') showed deformations into the external counter of epididymal ducts and reduction in spermatozoa number.
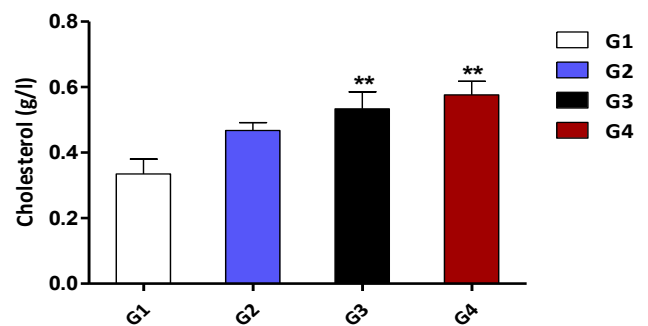

Figure 3 Evaluation of Cholesterol level (g/l) of rats exposed to mixture pesticide after eight weeks of treatment. Results are expressed as (Mean±SD, n=8). ** $P < 0.01$.

3.4. Effect of the mixture on Oxidative stress markers

Results showed a non-significant increase of MDA level in testes and epididymis tissues of G2 animals compared to G1, as shown in Table 2. Besides, in G3 and G4, a significant increase ($P < 0.01$, $P < 0.001$) of the MDA level in testis and epididymis tissues was observed compared to the control animals.

The pesticides mixture significant decreased the Gpx activity of testis and epididymis tissues in G3 and G4 groups compared to the control group G1. However, in the G2 group, we have found a non-significant decrease in the GPx level of testis and epididymis tissues compared to the control group G1.

GSH level in testes and epididymis is presented in Table 2. These results indicate that the treatment with a mixture of pesticides leads to the obtention of a highly significant decrease ($P < 0.01$) of GSH level in the G3 and G4 groups compared to the control group G1. On the other hand, the G2 group presented a non-significant reduction compared to the control one.

4. Discussion

Pesticides may directly damage spermatozoa, alter Sertoli and Leydig cell's function, or disrupt the endocrine function in any stage of hormonal regulation (hormone synthesis, release, storage, transport, and clearance; receptor recognition and binding) (Bretveld et al 2007).

The present study demonstrates a significant decrease in the mass of testis and epididymis associated with a decrease in testosterone level and the count, vitality, and

motility of spermatozoa in rats treated with mixed pesticides: Cypermthrine, Metalaxyl, and Mancozebas compared to the control group. It is suggested that the decrease in the testis weight is probably via two effects. The anti-spermatogenic is the first one that can decrease the diameter and number of spermatogonia, spermatocytes, and spermatids in the seminiferous tubules of the testis. The second one is the anti-androgenic effect which reduces the number of sperms in testis tubules (Sakr and Shalaby 2011; Smith and Walker 2014; Yağmur et al 2020). Moreover, this result is consistent with those obtained by Lucier et al (1977) after the treatment with the mixture of the fungicides Maneb and Zineb in male adult rats.

Table 1 Evaluation of sperm concentration (×10^6/ml), sperm motility (%) sperm viability (%) of rats exposed to mixed pesticide after eight weeks of treatment. Results are expressed as (Mean±SD, n=8). ** $P< 0.01$; *** $P< 0.001$.

Paramaters		G1	G2	G3	G4
Concentration (×10^6/ml)		83.71±6.70	72.73±4.19	57.59±3.15**	51.05±2.36***
Motility (%)		58.92± 3.14	56.93 ± 2.36	39.79 ± 4.83**	21.91±3.53***
Viability (%)	N	40.52±1.57	48.98±5.33	58.75±5.41*	74.67±7.32**
	A	24.04±4.25	19.94±1.95	11.38 ±1.22*	9.69±1.67**
	B	19.24±1.14	16.15±1.98	12.70±0.71**	9.57±2.63**
	C	16.20±2.77	14.62±4.59	4.41±1.46**	2.11±0.88**

Table 2 Mean Testes and epididymis levels of MDA (nmol/g tissue), GSH (nmol/mg proteins), and GPx (μmol GSH/mg proteins) of rats treated with mixture pesticide after eight weeks of treatment. Results are expressed as (Mean±SD n=8). * $P< 0.1$; ** $P< 0.01$; *** $P< 0.001$.

	MDA(nmol/g Tissue)		GSH(nmol/mg proteins)		GPx(μmol/GSH/mgproteins)	
	Testis	Epididymis	Testis	Epididymis	Testis	Epididymis
G1	0.21±0.02	0.16±0.03	37.86±2.05	21.06±1.58	0.20±0.016	0.14±0.011
G2	0.25±0.02	0.21±0.02	35.69±3.07	21.06±1.58	0.18±0.009	0.12±0.013
G3	0.40±0.03**	0.38±0.04**	22.05± 3.061**	16.3±0.82**	0.12±0.01*	0.08±0.01*
G4	0.42±0.04**	0.44±0.04***	18.78±2.98**	11.14± 1.34***	0.10±0.01**	0.04±0.01***

Similarly, other studies demonstrate the same effect after treatment animals with mancozeb. Sunder et al (2002) observed that the mixture of (Metalaxyl + mancozeb) caused alterations in testis tissues, a disruption of germinal epithelium, atrophy of the seminiferous tubules causing androgen insufficiency state, and atrophy of Leydig cells leads to decreased testis weight. It can also be probably due to the loss of histoarchitecture, where the atrophy of germ cells supported the reduction in organ weight (Desai et al 2016). Similar effects revealed the same data after oral intubation with Cypermthrin for only 30 days (Joshi et al 2010; Assayed et al 2008). It means that the reduction in testicular and epididymis mass could be linked to the histological changes in the male reproductive system (Jensen et al 2006). Our histological examination showed alterations in testicular tissue of rats after mixed pesticide's treatment as well as degeneration in somniferous tubules with loss of sperm, and reduction of spermatogenic cells number in some somniferous tubules, expansion of interstitial spaces, and induction of cell necrosis accompanied by apical sloughing in the section of testes in the treated groups as compared to the control groups (Elbetiha et al 2001; Ksheerasagar and Kaliwal 2003).

Our histologic examinations of the testes and epididymis sections (Figure 4) revealed that the used mixture of the pesticides induced in our experimental conditions in the seminiferous reduced spermatozoa in the lumen, especially in groups treated with the medium and high doses, demonstrating seminiferous tubule degeneration. The interstitial spaces are enlarged due to tubular atrophy, edema, and decreased sperms in the lumen. Similarly, the section of the epididymis showed deformations into the external counter of epididymal ducts and reduction in spermatozoa number as the compared control group.

This result is also in agreement with the study of Sharma and Singh (2010) that treating rats with cypermethrin could decrease epididymis mass by reducing sperm concentration and the degenerative change in epididymal tissues. Similarly, Khan and Sinha (1994) have reported that mancozeb could lead to the same effect by a decrease of sperm count and the increase of sperm with aberrant head morphology in the epididymis tube (Figure 5).

On the other hand, our research also showed reduced sperm motility after using a mixture of pesticides for eight weeks after treating rats. We hypothesize that the mixture has probably caused a morphological perturbation in the intermediate piece and the flagellum, responsible for spermatozoa movement. It cited that cypermethrin may affect sperm motility by altering enzymatic activities of mitochondria (oxidative phosphorylation); thus, it stops ATP formation responsible for the spermatozoa movement (Bedford 1983). This result agrees with the finding of Pant et al (1995), who indicated the same result after exposing rats to carbofuran, which belongs to the same family of mancozeb.
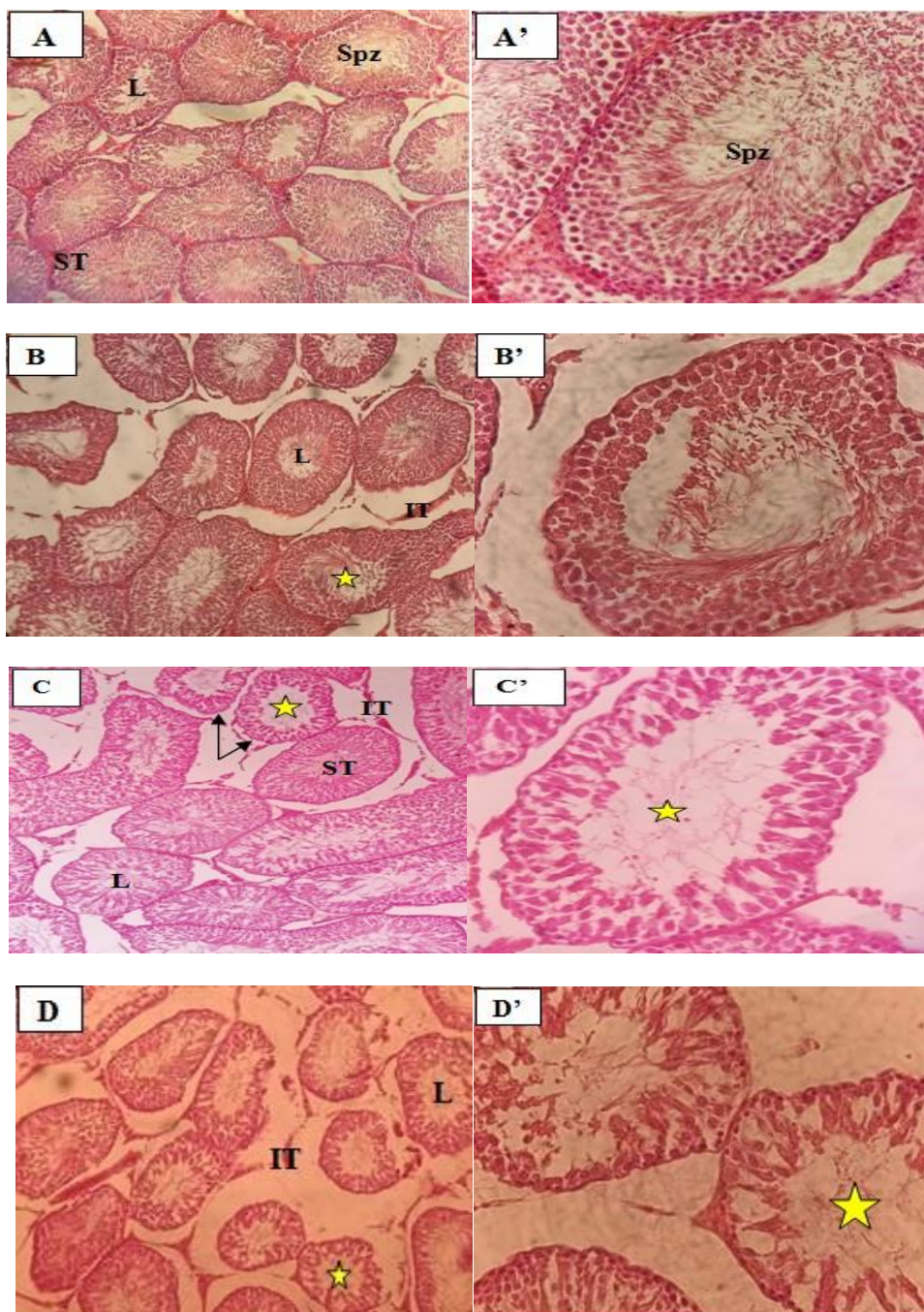
Figure 4 Photomicrography of transverse section in the somniferous tubules of the testes in the control and treated group (H.E.100X, 400X). Legend: ST: seminiferous Tubules; L: lumen; Spz: spermatozoa; Arrow: atrophic tubules; yellow star: a reduction in spermatozoid.

In terms of sperm viability, we found a decline in viable sperms collected in cauda epididymis after the treatment by mixed-used pesticides compared to the control group. Research showed that treatment with cypermethrin leads to a reduction in sperm vitality in the testis of rats. This is following the results of Singh et al (2014), who have noticed a drop in sperm vitality after exposing male rats to cypermethrin for 14 days.
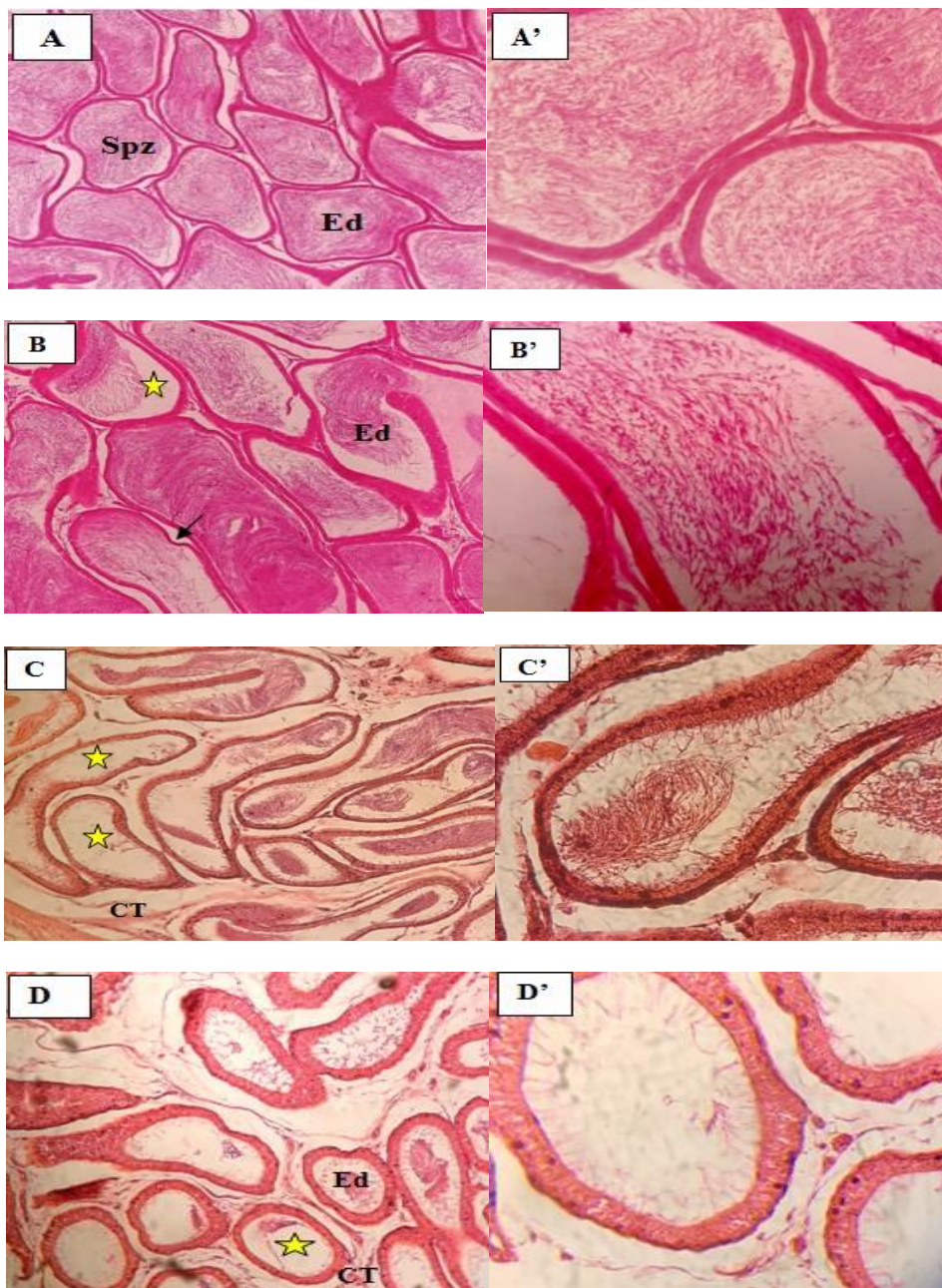

Figure 5 Photomicrographs of sections epididymis in all groups after the treatment with the mixture of pesticides compared to the control (H.E.100X, 400X). Legend: Ed: epididymis duct; arrow deformation; CT: connective tissue; yellow star: reduction in spermatozoid; Spz: spermatozoa.

Testosterone plays a clear and vital role in developing male reproductive tissues, especially the testis (Elbetieha et al 2001). The pesticides mixture significant reduces serum testosterone level in treated animals compared to the control group. The mixture of pesticides may probably affect the hypothalamus-pituitary axis, where the GnRH is produced, responsible for the liberation of LH. This hormone (LH) stimulates Leydig cells to produce testosterone; hence, a decrease in LH may lower testosterone levels (Joshi et al 2011). It has been further reported that pyrethroid insecticides which are the family of cypermethrin, can cause mitochondrial membrane fragility in Leydig cells and damage testosterone biosynthesis by diminished the transport of cholesterol into the mitochondria and reducing the transformation of cholesterol to pregnenolone in the cells, thus decreasing testosterone production (Zhang et al 2007).

This is in perfect concordance with our testosterone results and cholesterol concentration in the treated groups compared to no-treated rats.

The reproductive toxicity of pesticides may cause the low sperm count through the neuro-endocrine-mediated phenomenon and a hormone-disrupting property. This hypothesis suggested by Yousef et al (2003), leading to low sperm count, could be that pesticides interact competitively with androgen receptors and sex hormone-binding globulin, causing the endocrine system to disrupt. The present study indicated that the treatment with used mixed pesticides induces a decrease in sperm parameters: concentration, mobility, and vitality of spermatozoa. Concerning the rate vitality of spermatozoa in the treated animals, the data indicate a significant decrease in the live spermatozoa. Likewise, Sunder (2002) indicated that treating rats with a mixture of (metalaxyl + mancozeb) at the dose of 500 mg/kg for 30, 60, and 90 days induced a remarkable decrease in live sperm's percentage.

The disturbances in the reproductive system and quality of sperm might be attributed to oxidative stress (Agarwal and Said 2005). Reproductive organs are very susceptible to damage by free radicals due to their high content on polyunsaturated fatty acids (Noblanc 2011; Lopez 2007). Malondialdehyde (MDA) is the final result of lipid peroxidation operation that might be defined as an oxidative deterioration of polyunsaturated lipids (Debnath and Mandal 2000). Treating rats with mixed pesticides induced a significant increase in MDA levels in testis and epididymis tissues. It has been shown that the accumulation of cypermethrin in testicular and epididymis tissue led to membrane degeneration and extensive formation of free radicals.

Similarly, Sharma et al (2013) have found that the level of MDA was increased in the testis of rats after 14 days of treatment with pesticides. In addition, cypermethrin can affect mitochondria and stop the adenosine triphosphate generation, which leads to block the cell's main energy source, causing mitochondrial dysfunction and producing high concentrations of free radical (Joshi et al 2010). In another study, mancozeb can also increase MDA levels in testis and epididymis organs. Different studies have demonstrated that the toxicity of mancozeb is related to the association of its organic molecule to magnesium (Domico et al 2007). A different generation of ROS by Mn incorporates the oxidation of Mn2 to Mn3, which catalyzes DA oxidation with the production of toxicant reactive intermediaries (Diazveliz 2004). Kaloyanova et al (1991) reported that oxidative stress is one of the indications of metalaxyl-induced poisonousness.

Under oxidative stress, the concentration of glutathione peroxidase (GPx), which is supposed to act against the free radicals, changed in both testis and epididymis tissues. In the current study, Cypermthrin treatment was found to induce GPx reduction. This result agrees with Singh et al (2014), who has also deduced a reduction in GPx level in testis and epididymis tissue of rats

exposed to cypermethrin (3.83 mg/kg BW) for 14 days. Therefore, the reduction of GPx results from the decreased activity of glutathione (Sun 2007). Mancozeb can also cause the same result of Cypermethrine by impairment activities of those enzymes, indicating then the failure of the primary antioxidant system to act against free radicals (Ayala et al 2014). A decrease in glutathione (GSH) in testis and epididymis after exposure to the mixed pesticides in our experimental conditions could be related to the increase of GSH utilization because it acts with SH as a catalyst for the disulfide exchange reaction and finally contributes to (ROS) detoxification (Raina et al 2009). Other research explained that deltamethrin reduced the GSH activity in the testis (Singh et al 2014).

5. Conclusions

Exposure to a combination of pesticides by alimentation for 60 consecutive days may induce reproductive toxicity in male rats manifested by decreases in the fertility index, the weight of the sexual organs, semen characteristics, serum testosterone, and cholesterol, as well as severe histological changes. Also, it resulted in lipid peroxidation and depletion of antioxidant enzymes in the testes and epididymis of the rat. These results highly impose the necessity for more itemized testing of the toxicity of pesticide mixture exposure, especially by alimentation, and caution should be exercised in their handling as lengthy exposure time might lead to damaging health effects.

Conflict of Interest

The authors declare that there is no conflict of interest with this work.

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