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Investigation of the subchronic effects of low-dose pesticide mixture on rat testes

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Abstract

Pesticides are chemical agents used against living things such as insects, rodents, and weeds that cause toxicity in various tissues, including reproductive organs. In order to increase the effectiveness of pesticides, active ingredients are mixed with various formulations. The current study tried to determine the effects of pesticides and their mixtures on testicular tissue in rats. For this purpose, chlormequat chloride (CCC), pirimiphos methyl (PMM), glyphosate (GLY), tebuconazole (TBZ), chlorpyrifos methyl (CPM), deltamethrin (DLM), and their mix (acceptable daily intake (ADI) and ADIx10) were administried to Sprague-Dawley rats for 90 days. As a result of the examinations, the ADIx10 group showed the most damaging effect by showing a statistical difference in terms of sperm motility and membrane integrity. The GLY group had a similar effect on membrane integrity as the ADIx10 group, while it had a moderately detrimental effect on motility. Degenerations of spermatocytes, necrosis, and edema in intertubular spaces were observed in all pesticide groups. Similarly, 8-hydroxyguanosine (8-OHdG) expression and Caspase-3 expression were moderate in all pesticide groups. Moreover, 8-OHdG expression and Caspase-3 expression were higher in the ADI and ADIx10 groups compared to other groups. As a result, pesticides and their mixtures cause histopathological changes in the testicular tissue of rats and decrease sperm quality.

Key Words: Oxidative stress, pesticide, rat, sperm, testis

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Introduction

One of the chemicals that cause environmental pollution is pesticides[16]. Many people are exposed to pesticides commonly used against pests and weeds in modern agriculture[24]. The American Chemical Society's list introduces 2,500,000 new chemical compounds every year. It is difficult to distinguish between the environmental burden of these chemicals and their toxic effects on human health[25]. Moreover, toxicity assessments for a single chemical may not adequately describe all risks, such as genotoxicity, endocrine disruptors, and organ toxicity. In practice, the population is never exposed to a single chemical[56]. When two or more chemicals come together, besides potentiating or inhibiting protective mechanisms, their effects can occur in a synergistic, combined, or competitive way, and it is a fact that unidentified risks and hazards can occur when testing single compounds[38]. There are particular concerns about toxicities occurring in different tissues; for example, neurotoxicity[9,17,66], reproductive toxicity[21], cardiotoxicity[49,65], nephrotoxicity[60], hepatotoxicity[27,57], genotoxicity[53] and endocrine disruptions[11,18,26,31,45].

Pesticides are chemicals that cause the fertility rate to decrease in males and females[22]. Pesticides cause histopathological changes in testicles, a deterioration in spermatogenesis, a decrease in testosterone level, a deterioration in sperm morphology, and a decrease in sperm motility in rats[67]. In addition, a male with pesticides in his blood could theoretically infect the female uterus by releasing pesticide-laden sperm into the vagina during sexual intercourse[15]. Although pesticides provide great benefits to human life, they have negative effects on the environment and human health[44]. Pesticides affect the male reproductive system by causing direct damage to cell structure, changing the DNA structure, or by changing gene expression[59]. Moreover, pesticides cause oxidative damage to females and damage to reproductive physiology[13]. Although decreased fertility has been observed among men exposed to pyrethroids, organophosphates, and carbamates in greenhouses, no other pesticide has been found to have such dramatic effects on male fertility[51]. Exposure of a pregnant female rat to pesticides resulted in decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility in the F1 generation. Decreased sperm concentrations were also found in the male pups of these male rats, and it was stated that they could act transgenerationally by an epigenetic mechanism epigenetic-mediated mechanisms[6]. Studies have shown an increase in Caspase-3 and 8-OHdG expression levels with subacute exposure to pesticides[39].

The purpose of this research was to investigate the impact of subchronic pesticide exposure and their combinations on the quality of sperm and histopathological changes in testicular tissue of rats.

Materials and Methods

Ethical approval: The approval of the Atatürk University Animal Experimentations Local Ethics Committee (protocol number: 2021/74) was obtained for the current study.

Preparation of experimental animals: In this study, male Sprague Dawley rats, 3 months old were used. Animals were kept in the Atatürk University Medical Experimental Application and Research Center under standard conditions (25±2 °C, 45±5% humidity, and 12 hours of light/dark cycle). The animals adapted to the environment for a week. The rats were divided into nine groups: Control group, Chlormequat chloride (CCC) group, Pirimiphos methyl (PMM) group, Glifosat (GLY) group, Tebukonazole (TBZ) group, Klorpirifos -Metyl (CPM) group, Deltametrine (DLM) group, acceptable daily intake (ADI) group and ADIx10 group.

Preparation of chemicals: The compounds were chosen based on a set of criteria that included the scope of use, existing health problems, and the potential for toxicokinetic or toxicodynamic application that could result in synergism or other interactions, increasing toxicity, or causing

unexpected toxicological responses. The ADI for each chemical in the combination was the recommended dosage. The ADI is the maximum dose of a drug, expressed as mg/kg body weight, that can be consumed every day for the rest of one's life without posing any health risks. Common cumulative toxicity can develop when many chemicals are exposed to similarly low levels. Exposure of rats was monitored according to Regulation 440/2008/EC, the OECD GD 452 (OECD, 2009) method for chronic toxicity, the most complete guideline for testing the chronic toxicity of chemicals, which will simulate lifetime exposure for human[55]. Selected doses were adjusted to ADI and ADIx10 (mg/kg), which are considered safe doses for humans[62].

Application of the experimental model: After the acclimatization period, the amount of feed per animal and water consumption were calculated. From the 0th day (week 1), the mixture prepared in the doses specified in corn oil was added to the feeds and the amount of feed consumed every day was weighed. At the same time, daily water consumption was monitored. On the 90th day, the experiment was terminated and the animals were sacrificed under sevoflurane anesthesia and the tissue tissues were removed.

Experimental groups: Chlormequat chloride (CCC): 0.04 mg/kg bw; Pyrimiphos methyl (PMM): 0.004 mg/kg body weight; Glyphosate (GLY): 0.5 mg/kg bw; Tebuconazole (TBZ): 0.03 mg/kg body weight; Chlorpyrifos methyl (CPM): 0.01 mg/kg bw; Deltamethrin (DLM): 0.01 mg/kg bw and the active ingredient mixture group ADI and ADIx10 were prepared from the active ingredients containing CCC, PMM, GLY, TBZ, CPM and DLM. Soluble concentrate (SC) formulation containing 460 g CCC per liter, emulsifiable concentrate (EC) formulation containing 500 g PMM per liter, SC formulation containing 441 g GLY per liter, powder formulation containing 600 g TBZ, EC formulation containing 227 g CPM per liter, SC formulation containing 50 g DLM per liter, ADI and ADIx10 doses were prepared, and formed. The pesticides were dissolved in corn oil in accordance with the OECD guidelines and added to the feed. A separate cage was provided for each animal, and the feeds were re-prepared every day and the consumption amounts were measured per animal.

Collection and evaluation of sperm samples: The testicles of the sacrificed rats were removed, and the epididymis were separated from the testis. The cauda epididymis was taken into a petri dish to be used in semen analysis and kept in 5 mL of physiological water (0.9% sodium chloride solution). The right cauda epididymis was trimmed in this solution, and the spermatozoa were allowed to pass into the liquid. A contrast microwave with a heating plate (Leica Germany) was used for sperm evaluation. The slide was placed in a phase contrast microscope with a heating plate, the semen sample was left on the slide and covered with a coverslip, and the percentage of sperm motility was calculated. Three different microscope fields were examined for sperm motility predictions and were determined as the "motility score"[4]. For sperm membrane integrity, the semen sample was mixed with the eosin nigrosine dye (1.67% eosin, 10% nigrosine and 2.9 g sodium citrate) on a slide. Frotting was taken from the mixture and left to dry. Slides were then evaluated with a microscope at 20x magnification. Depending on the staining status of the sperm heads, they were classified as damaged or not. For each animal, 300 sperm cells were examined and the ratio was expressed as a percentage (%)[3].

Histopathological examination: Following standard tissue procedures, samples were embedded in paraffin blocks and fixed in 10% formaldehyde solution. Slides for histopathological examination were stained with hematoxylin and eosin (HE). Slides were examined with a light microscope (Olympus BX 51, Japan). Sections were interpreted as absent (0), mild (1), very mild (2), moderate (3), very (4), and severe (5) for histopathological examination[37].

Immunohistochemical examination: Sections taken on adhesive (poly-L-lysine) slides were kept in 3% H_2O_2 for 10 minutes. Then the slides were

Table 1. Spermatological and histological parameters of the study groups (x±SEM)

	Control	CCC	PMM	GLY	TBZ	CPM	DLM	ADI	ADIx10
Total Motility	71,25±6,29^{e}	45,00±5,77^{cd}	46,25±7,50^{d}	43,75±7,50^{cd}	33,75±8,53^{bc}	45,00±5,77^{cd}	51,25±8,53^{d}	26,25±8,53^{b}	8,75±4,78^{a}
Sperm Membrane Integrity	60,00±4.08^{e}	32,50±2,88^{bc}	40,50±3,31^{bcd}	17,75±6,34^{a}	46,25±4,78^{d}	42,50±2,88^{cd}	32,50±2,88^{bc}	31,25±8,53^{b}	8,25±2,36^{a}

a-e; Values indicated by different letters in the same column are significantly different from each other ($P < 0.05$).

boiled in a 1% antigen retrieval citrate buffer (sodium citrate dihydrate, 0.0874 M, citric acid, 0.0126 M, pH = 6.1) solution and allowed to cool at room temperature. Tissues were incubated with protein block (goat serum, Cat no: ab7481, UK) for 5 minutes. Primary antibodies were then added to the tissues (8-OHdG Cat No.: sc-66036, 1/100 dilution, USA; Caspase-3 Cat No: sc-56053, 1/100 dilution, USA) and incubated at 37°C for one hour. 3–3' Diaminobenzidine (DAB) chromogen was used on the slides. The stained sections were interpreted using an light microscope (ZEISS, Germany)[48].

Statistical analysis: The analysis of the data obtained from the study was conducted using the SPSS 26.0 program. In the study, spermatological data were presented as mean value SEM. Spermatological parameters were analyzed using a one-way analysis of variance test and a post-hoc Tukey test. The comparison of groups in histopathological examinations was done with the Duncan test. The nonparametric Kruskal-Wallis test was used to detect group interaction, and the Mann-Whitney U test was used to determine the difference between groups. Five areas were randomly selected from the immunohistochemically stained images and evaluated in the ZEISS Zen Imaging Software program. For % area, data were statistically defined as mean and standard deviation (mean SD). One-way ANOVA followed by Tukey's test was performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls. As a result of the test, a value of $P < 0.05$ was considered significant and the data were presented as mean ± SD.

Results

Sperm analysis results: Sperm analysis results were presented in Table 1. The total motility value decreased significantly ($P < 0.05$) in the ADI and ADIx10 groups. In addition a decrease in total motility was observed in the CCC, PMM, GLY, TBZ, CPM, and DLM groups compared to the control group ($P < 0.05$). While the highest spermatozoa membrane integrity was seen in the Control group, a statistically significant difference was observed between the other groups ($P < 0.05$). Plasma membrane integrity ratio decreased significantly in the ADIx10 and GLY groups compared to other experimental groups ($P < 0.05$). There was a significant difference in plasma membrane integrity rate in the other pesticide groups compared to the control group ($P < 0.05$).

Histopathological findings: Histopathological evaluations are presented in Table 2. When the testicular tissues in the control group were examined histopathologically, it was seen that the tissues had a normal histological structure. In the CCC group, severe degeneration of the spermatocytes in the seminiferous tubular walls of the testis tissue and thinning due to moderate necrosis, severe edema in the intertubular areas, and severe congestion in the interstitial vessels were observed ($P < 0.05$). In the PMM group, mild degeneration and thinning as a result of mild degeneration of spermatocytes in the tubular wall in the testis tissue, mild edema in the intertubular

Table 2. Histopathological findings of testicular tissues

	Degeneration of spermatocytes	Necrosis of spermatocytes	Edema in the intertubular spaces	Congestion of vessels
Control	0	0	0	0
CCC	+4	+3	+4	+3
PMM	+2	+1	+2	+3
GLY	+4	+3	+4	+4
TBZ	+4	+3	+4	+4
CPM	+3	+3	+3	+3
DLM	+3	+2	+3	+3
ADI	+4	+4	+4	+4
ADIx10	+5	+5	+5	+5

In the study, blind pathology method was used in histopathological analysis, evaluations were made by 2 expert pathologists and the results were recorded. We do not have a quantitative evaluation program that we use in histopathological examinations. However, quantitative calculations were made in immunohistochemical examinations and the values are presented in the table.

areas, and moderate congestion in the interstitial vessels were observed. A statistically significant difference was found between the ADI groups and the PMM group ($P < 0.05$). In the GLY group, severe degeneration of the spermatocytes in the seminiferous tubule wall and thinning as a result of moderate necrosis, severe edema in the intertubular spaces, and severe congestion in the vessels in the interstitial region were observed ($P < 0.05$). In the TBZ group, severe degeneration of spermatocytes in the seminiferous tubule wall and thinning due to moderate necrosis, severe edema in the intertubular regions, and severe congestion in the interstitial vessels were observed ($P < 0.05$). In the CPM group, moderate degeneration and thinning as a result of necrosis in spermatocytes in the seminiferous tubule wall, moderate edema in the intertubular regions, and moderate congestion in the interstitial vessels were observed ($P < 0.05$). In the DLM group, moderate degeneration of the spermatocytes and thinning as a result of mild necrosis in the seminiferous tubule wall, moderate edema in the intertubular regions, and moderate congestion in the interstitial vessels were observed. A statistically significant difference ($P < 0.05$) was detected when compared with the ADIx10 group. In the ADI group, severe degeneration and thinning due to necrosis in spermatocytes in the seminiferous tubular wall, severe edema in the intertubular areas and severe congestion in the interstitial vessels were observed. In the ADIx10 group, severe degeneration and thinning due to necrosis in spermatocytes in the seminiferous tubule wall, very severe edema in the intertubular spaces, and very severe congestion in the interstitial vessels were observed (Fig. 1).

Table 3. Scoring of immunohistochemical findings of testicular tissues

	8 OHdG expression (W/sr)	Caspase 3 expression (W/sr)
Control	17.59±0.12^{a}	20.07±0.03^{a}
CCC	67.06±2.47^{b}	74.43±2.71^{b}
PMM	43.19±1.39^{c}	29.1±0.26^{c}
GLY	83.57±2.97^{d}	75.3±1.19^{b}
TBZ	69.84±1.64^{b}	75.48±1.45^{b}
CPM	86.44±3.18^{d}	77.59±2^{b}
DLM	66.66±3.33^{b}	42.87±1.57^{d}
ADI	85.19±3.27^{d}	99.56±3.2^{e}
ADIx10	124.48±3.94^{e}	132.44±3.48^{f}

a,b,c,d,e,f; different letters in the same column represent statistically significant difference ($P < 0.05$).
Abbrevations; W/sr (unit of luminous intensity)

Immunohistochemical findings: Immunohistochemical findings are presented in Table 3. Expressions of 8-OHdG and Caspase-3 in testicular tissues in the Control group were evaluated as negative. Moderate expression of

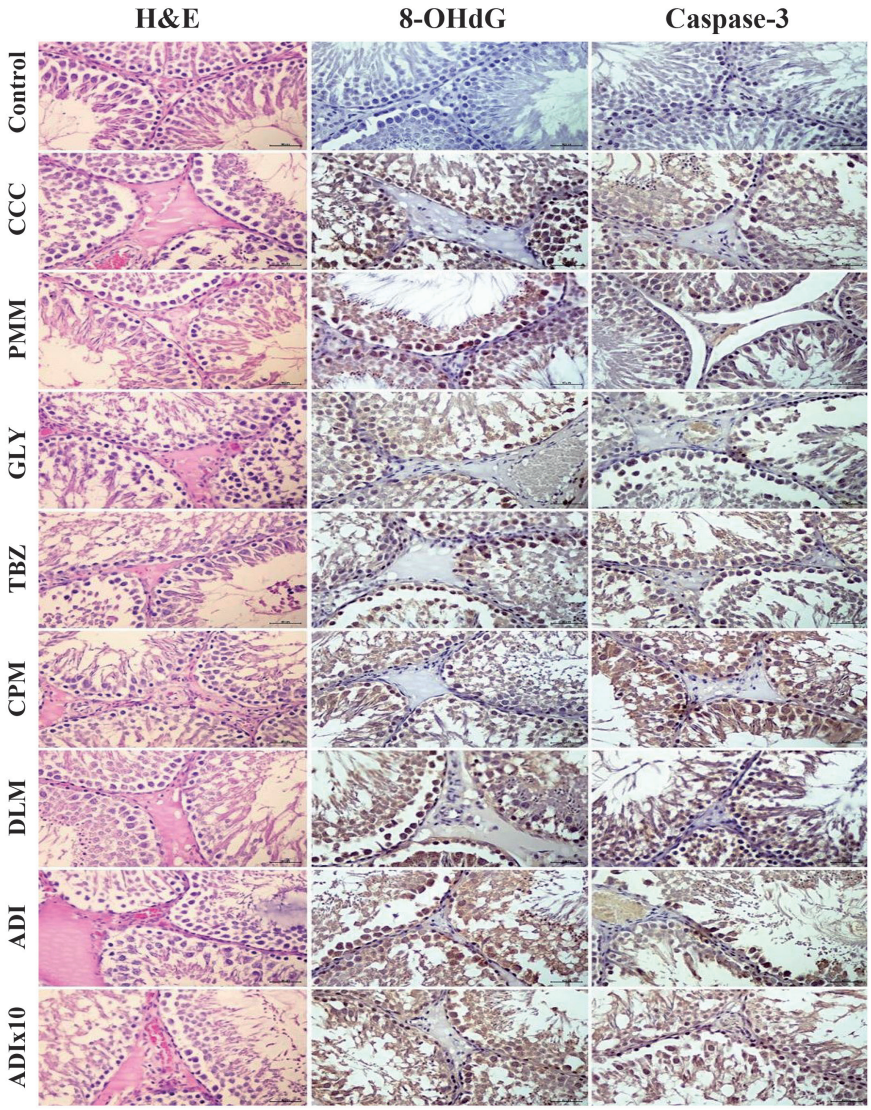

Fig. 1. Testicular tissue, histopathological findings (H&E), 8 OHdG and Caspase 3 expressions in spermatocytes and spermatogonia. IHC-P, Bar: 20 µm.
Abbrevations; CCC: Chlormequat chloride, Pirim: Pirimiphos-methyl, GLF: Glyphosate, TBZ: Tebucanazole, Chlor: Chlorpyrifos-methyl, Delta: Deltametrin, IHC-P: Immunohistochemical paraffin protocol.

8-OHdG and Caspase-3 were detected in the cytoplasm of spermatocytes and spermatogonial cells in the CCC group. In the PMM group, mild 8-OHdG and very mild Caspase-3 expressions were observed in the cytoplasm of spermatocytes and spermatogonial cells, and a statistically significant difference ($P < 0.05$) was detected when compared with the ADIx10 group. In the GLY group, severe 8-OHdG and moderate Caspase-3 expression were detected in the cytoplasm of spermatocyte and spermatogonial cells. Moderate expression of 8-OHdG and Caspase-3 was observed in the cytoplasm of spermatocyte and spermatogonial cells in the TBZ group. In the CPM group, strong levels of 8-OHdG and moderate Caspase-3 expressions were detected in the cytoplasm of spermatocyte and spermatogonial cells. Moderate expression of 8-OHdG and Caspase-3 was detected in the cytoplasm of spermatocyte and spermatogonial cells in the DLM group, and a statistically significant difference ($P < 0.05$) was detected when compared to the ADIx10 group. In the ADI groups, severe 8-OHdG and Caspase-3 expressions were observed in the cytoplasm of

spermatocyte and spermatogonial cells. In the ADIx10 group, very severe 8-OHdG and Caspase-3 expressions were detected in the cytoplasm of spermatocyte and spermatogonial cells (Fig. 1).

Discussion

Pesticides are grouped as insecticides, fungicides, herbicides, rodenticides, molluscicides, and nematicides[12]. Pesticides reduce agricultural products losses and play an important role in agricultural development as they can improve affordable product and food quality[5,20,54]. Pesticides are pollutants of the environment and can be found in samples from water, air, soil, animal, and human tissues[16]. Studies have reported that pesticides also have a negative effect on the male reproductive system[33,41,47]. This study investigates germ cell damage caused by pesticides and pesticide combinations histologically and oxidative stress by the immunohistochemical method.

The plant protection product contains active ingredients and excipients. Every pesticide purchased from the dealer is a formulation. Pesticide formulations allow the active ingredient to adhere to the plant surface. In addition to this situation, it is the excipients that make the difference between the active substance and the formulations and it is a necessity to investigate their possible toxic effects[34]. Due to the large number of pesticide combinations that can be found in the ecosystem, it is not possible to physically test the effects of these mixtures on living things[43]. We investigated the effects of pesticide active ingredients and their mixtures on the male reproductive system with the experimental groups we created in our study. This study examines the effects of prolonged exposure (3 months) to very low doses of ADI and ADIx10 levels of a cocktail of six chemicals with which humans are in frequent contact, on various biological parameters in an animal model.

CCC is a widely applied plant growth regulator that inhibits unwanted shoots[29]. In a study with pubertal exposure to CCC, also known as chlorocholine chloride[28], it was determined that CCC did not cause any changes in rat testis tissue, but in our study, in the CCC group, spermatocytes on the wall of the seminiferous tubules in the testis tissue, degeneration, necrosis and edema were determined. This suggests that acute CCC administration is more toxic than pubertal CCC. It may be due to the recovery and restoration of the effect of CCC in testicular tissues after pubertal CCC administration. Pubertal CCC exposure caused a decrease in sperm motility, and a significant decrease was observed in sperm motility in our study ($P < 0.05$). In this respect, the results are similar. DLM is a widely used broad-spectrum synthetic type II pyrethroid insecticide [α-cyano-3-phenoxybenzyl-(1R,S)-cis, trans-3-(2,2-dibromovinyl) 2,2-dimethylcyclopropane-carboxylate] and protects vegetables, fruits and agricultural produce against pests such as mites, ants, insects and weeds[2]. Pyrimiphos-methyl is a broad-spectrum organophosphate insecticide widely used as a cereal preservative globally. So far, pyrimifos-methyl has proven to be very effective against a wide variety of stored product insect pests[30,36,50]. It was stated in a study that the combination of DLM and DLM+Ridomil decreased the sperm count, sperm motility and serum gonadotropins of rats[19]. DLM 1% + Chlorpyriphos 35% EC has been shown to directly or indirectly inhibit sperm production and motility[52]. In another study, the diameter of the seminiferous tubule per unit area decreased and a thickening of the basement membrane was with determined in the histomorphological examination of testicular tissue of rats administered deltamethrin[40]. In another study prepared with a mixture of DLM and dimethoate+DLM in male mice, significant decreases were found in sperm count, viability and motility addition, with a significant increase in spermatozoa with abnormal morphology[1]. These results are in agreement with our study. The herbicide GLY, N-(phosphonomethyl) glycine, is a biocide with broad spectrum activity introduced in 1974 for weed control in agricultural production areas[58]. In a study, it was determined that GLY caused a decrease in membrane integrity in sperm motility and caused degeneration in Sertoli cells[8]. Similar results were found in our study,

and membrane integrity in the GLY group was significantly decreased compared to the other groups ($P < 0.05$). Triazole fungicides are one of the top ten classes of pesticides used today and have higher consumption compared to other fungicides available worldwide, with TBZ being one of the members of this group[68]. Chlorpyrifos-methyl (O, O-dimethyl O- (3,5,6 - trichloro - 2 - pyridinyl) -phosphorothioate), which has a similar molecular structure but higher water solubility and vapor pressure than chlorpyrifos, is one of the most widely used insecticides in the World[42]. In a study conducted with TBZ in rats, it was reported that chlorpyrifos it caused a decrease in sperm motility, an increase in sperm count and sperm abnormalities. Histopathologically it also caused deterioration in testicular tissue[10]. In one study with TBZ without a statistical difference, a decrease in sperm count in the testis was determined. In the same study, a decrease in epididymal sperm count, testosterone level, increase in superoxide dismutase (SOD) level and decrease in testicular total protein level were found[64]. In our study, a decrease in motility and a decrease in sperm membrane integrity were found in the TBZ group ($P < 0.05$). In a study of PMM in male rats, it was reported that pesticides caused a significant decrease in sperm density and sperm motility[47]. CPM causes decrease in sperm count, decrease in motility, increase in abnormal sperm count and histopathological changes in rats[70]. This result is similar to our study. Histologically, moderate changes were observed in the cyhorpmrifos methyl group. In rats treated with PMM, a decrease in sperm count and motility and an increase in abnormal sperm count were observed. In addition, histologically, it showed that there was enlargement of the interstitial space, inhibition of spermatogenesis, sparseness of Leydig cells and edema in the testicles in the experimental groups compared to the control group[47]. In our study, histological changes were less in the PMM group compared to the other experimental groups. However, sperm quality was similar to other pesticide groups.

Mixed pesticides cause synergistic toxicity in animal models[61,69,70]. In a study showing the effects of pesticide mixtures in male rats, sperm count and quality decreased, prostate and epididymis weight decreased, testicular weight increased and histopathological changes occurred[32]. In another study, while testicular and epididymis weights, testosterone levels and sperm quality decreased in rats treated with pesticide mixture, malondialdehyde (MDA) level increased[14]. In other studies, low-dose pesticide mixtures caused oxidative damage and testicular dysfunction[7,23,70]. In our study, testicular damage, increased oxidative stress and consequently decreased sperm quality occurred in all pesticide groups. Our findings are compatible with the obtained literary data.

Endogenously or exogenously produced reactive oxygen species (ROS) can attack the lipid membrane, protein profile and nucleic acid in living cells[63]. Oxidized guanosine can become incompatible with adenosine instead of cytosine and cause a mutation in the gene. Therefore, 8-OHdG can be used as a biomarker of DNA damage[46]. Caspases enter the active (cleaved) form in cells undergoing apoptosis and play a very important role in the transduction of apoptotic signals in dying cells[35]. 8-OHdG expression and Caspase-3 expression were higher in all experimental groups compared to the control group. This situation reveals that pesticides increase DNA damage in testicular tissue and cause apoptosis. 8-OHdG expression Caspase-3 expression was found to be higher in ADI and ADIx10 groups compared to other groups. Similarly, degeneration of spermatocytes, necrosis of spermatocytes, edema in the intertubular spaces, congestion of vessels were found mostly in ADI and ADIx10 groups. In general, the lowest sperm motility and membrane integrity were observed in ADIx10 and ADI groups, while a statistically significant difference was found between the other groups. This proves that the mixture of pesticides has more negative effects on testicular tissue. As a result, pesticides cause histological deterioration in testicular tissue, decrease in sperm quality, increase in oxidative stress and apoptosis in testicular tissue.

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