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THE HISTOPATHOLOGICAL INVESTIGATION OF LIVER IN EXPERIMENTAL ANIMALS AFTER SHORT-TERM EXPOSURES TO PESTICIDES

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Abstract

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Pesticides are one of the most dangerous pollutants for human and environment health due to their toxic effects and accumulation features. Acetamiprid (Acm), a neonicotinoid insecticide, and Propineb (Pro), a dithiocarbamate fungicide, are also widely used to control sucking insects and fungal infections on crops, respectively. The present study was undertaken to investigate the histopathology of Acm, Pro and the mixture of them were investigated on the liver tissue of mice. Fifteen groups were prepared for the histopathological examinations. Three of these were the positive, and the other three were the negative control groups. Nine of these were experiment groups. The mice were intaperitoneally treated to three different concentrations of pesticides for short-term (24 and 48 h) treatments. The histopathological effects of pesticides on the liver tissue of mice were determined by light microscopy. After 24 and 48 h, all concentrations of Acm significantly induced formation of sinusoidal dilatation. Vacuolar degeneration was also significantly observed at the lowest concentration (0.625 μg/ml) of Acm for 48 h. Pro (25 and 50 μg/ml) significantly caused vacuolar degeneration in the hepatocytes and sinusoidal dilatation in the parenchyma for 24 h. It was also observed that the mixture of Acm and Pro significantly induced formation of vacuolar degeneration, vascular dilatation and sinusoidal dilatation in almost all of the concentrations both 24 and 48 h when compared with the negative control. Furthermore, the mixture of Acm and Pro also significantly produced synergistic effects when the results of individually applied pesticides compared to their mixture at the same concentrations. These results led to the conclusion that Acm and Pro may have destructive effects on the liver tissue of mice. So, the use of these pesticides should be under control in agricultural fields.

Key words: Acetamiprid; histopathological alterations; mice; mixture of pesticide; Propineb
Abbreviations: Acm – Acetamiprid; i.p. – intraperitoneal; MMC – Mitomycin C; Pro – Propineb

Introduction

Nowadays, the widespread use of pesticides in agricultural areas all over the world poses a threat to all living creatures and environment due to their toxic effects and accumulation of their hazardous features. Furthermore, pesticides rapidly spread around through various agents such as water, air, and food chain.

Neonicotinoids are crucially potent neurotoxic insecticides that act as agonists on the nicotinic acetylcholine receptors (Tomizawa and Yamamoto, 1993). Acm, a neonicotinoid insecticide, is widely used to control sucking insects on crops. Dithiocarbamate fungicides are mainly used for eradication of fungal infections on fruit, plants and vegetables (Soloneski et al., 2003). Pro, a dithiocarbamate fungicide is commonly used in the control of diseases in a wide range of crops in agriculture. Acm and Pro are commonly used on agricultural crops such

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as tomato, potato, melon, apple, tobacco, either separately or in combination. It is well known that the mixture of chemicals may be considerably more toxic than those used alone.

Histopathology is a critical part of the toxicologic and risk assessment of foods, drugs, chemicals, biologics, and medical devices. It is important that the approach to histopathologic examination satisfy regulatory demands for unbiased observations while facilitating the sensitive and efficient evaluation of large amounts of microscopic information (Crissman et al., 2004). Therefore, determination of the histopathologic effects of pesticide mixture by using different organisms is noteworthy in environmental studies.

Investigation of histopathological changes in animal tissues is a sensitive and rapid method, commonly used to detect effects of pesticides on various tissues and organs and to evaluate toxic potential and risk assessment of chemicals in the environment (Travlos et al., 1996; Bernet et al., 1999; Adams, 2002; Crissman et al., 2004; Mela et al., 2007). Although there are a few studies on the histological effects of Acm (Păunescu et al., 2011; Zhang et al., 2011, 2012) and Pro (Deveci et al., 1999; Guven et al., 1998, 1999), there is no investigation on the histopathological effects of mixtures of Acm and Pro on the liver of mice in the literature yet. The present study was conducted to investigate the histopathological effects of Acm, Pro, and their mixtures on the liver of mice *in vivo* due to the widespread use of these pesticides and lack of studies on their histopathological effects.

Material and Methods

Test chemicals

In the present study, the commercial formulation of Acm (containing 20% Acm as active ingredient) (CAS No: 135410-20-7) and Pro (containing 70% Pro as active ingredient) (CAS No 12071-83-9) were used as the test materials. The chemical structures of Acm and Pro are shown in Figures 1 and 2. Mitomycin C (MMC; CAS No, 50-07-7) was obtained from Sigma® (Taufkirchen, Germany).

MMC was used as a positive control, as it was previously confirmed as a mutagenic agent (Giri et al., 2002). Moreover, distilled water was used as the negative control. All test solutions were prepared just before each experiment.

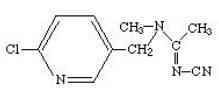

Fig. 1. Chemical structure of acetamiprid

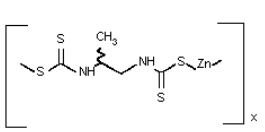

Fig. 2. Chemical structure of propineb

Concentration administration and sampling times

The intraperitoneal (i.p.) application is favored since it is one of the fastest and most efficient means of delivering test chemicals in a short-term assay and it has been used by many researchers (Azarnia et al., 2008; Bakare et al., 2009; Al-Qudsi and Linjawi, 2012).

The concentrations used in this study were selected according to the results of a preliminary study. In the preliminary study, the concentrations were selected on the basis of doses used against diseases in crops such as tomato, potato, melon, apple, and tobacco (Karaca et al., 2009). The concentrations of mixtures of Acm and Pro that were dissolved in water (0.625 + 12.5; 1.25 + 25; 2.5 + 50; 5 + 100; 10 + 200) µg/ml were used. In the preliminary study, it was observed that the mixtures of Ace and Pro exhibited high cytotoxic effects in their two highest concentrations (5 + 100; 10 + 200) µg/ml and decreased the ratio of dividing cells at these concentrations in 48 h treatment period. Based on the cytotoxicity of the test chemicals, the first three concentrations (0.625 + 12.5; 1.25 + 25; 2.5 + 50) µg/ml were determined as the concentrations to be tested in this study. In addition, pesticides were also tested separately in order to determine whether these pesticides would become effective or not when they are alone.

The animals

In this study, mice (*Mus musculus*), (8–10 weeks of age, with average body weight of 20–25 g), were used and purchased from Abant Izzet Baysal University Experimental Animals Applications and Research Center, Turkey. The animals were maintained in closely inbred colony under conventional laboratory conditions at a room temperature of 25±5°C and in 12 h dark and 12 h light cycles. Food pellets and water were provided ad libitum. The experiment was approved by the Ethics Committee of Abant Izzet Baysal University in Turkey (The Ethics Committee decision no was B. 30.2.ABÜ.0.05.05-050.01.04-64). 180 mice were randomly allocated into fifteen groups (n = 6 per group for 24 h; n = 6 per group for 48 h). The groups were as follows:

- Group I (negative control) was treated with distilled water both 24 and 48 h.
- Group II (positive control) was treated with MMC (0.2 µg/ml) both 24 and 48 h.
- Group III was treated with 0.625 µg/ml concentration of Acm both 24 and 48 h.
- Group IV was treated with 1.25 µg/ml concentration of Acm both 24 and 48 h.
- Group V was treated with 2.50 µg/ml concentration of Acm both 24 and 48 h.
- Group VI was treated with distilled water both 24 and 48 h.

• Group VII was treated with MMC (0.2 µg/ml) both 24 and 48 h.

• Group VII was treated with 12.5 µg/ml concentration of Pro both 24 and 48 h.

• Group IX was treated with 25 µg/ml concentration of Pro both 24 and 48 h.

• Group X was treated with 50 µg/ml concentration of Pro both 24 and 48 h.

• Group XI was treated with distilled water both 24 and 48 h.

• Group XII was treated with MMC (0.2 µg/ml) both 24 and 48 h.

• Group XIII was treated with (0.625 + 12.5) µg/ml the mixture of single concentrations of Acm and Pro both 24 and 48 h.

• Group XIV was treated with (1.25 + 25) µg/ml the mixture of single concentrations of Acm and Pro both 24 and 48 h.

• Group XV was treated with (2.50 + 50) µg/ml the mixture of single concentrations of Acm and Pro both 24 and 48 h.

Preparation of tissue samples and examination

In the present study, mice were *i.p.* once injected with Acm and Pro at different concentrations of (0.625, 1.25 and 2.50 µg/ml; 0.01 ml per gram of each animal) and (12.5, 25 and 50 µg/ml; 0.01 ml per gram of each animal) for 24 and 48 h, respectively. In addition, the mixture of single concentrations of Acm and Pro was used in the test concentrations of the pesticides in the same treatment periods. The animals were sacrificed by cervical dislocation after treatment periods, they were quickly dissected and their livers were removed.

For histopathological examination, the liver tissues were dissected and the tissue samples were fixed at 10% buffered formaldehyde for 24 h, processed using a graded ethanol, xylene and paraffin series, and embedded in paraffin. The paraffin sections were cut into 4 mm thick slices and stained with hematoxylin and eosin for light microscopic examination. The sections were viewed and photographed using a Nikon light microscope (Nikon Eclipse i80, Tokyo, Japan) with an attached photographic machine (Nikon DS-Fi1, Japan). Three slides were prepared from each liver. All sections were evaluated for the degree of basophilic stippling, portal mononuclear cell infiltration, granulated cytoplasm, eosinophilic debris, vacuolar degeneration, sinusoidal dilatation, and vascular congestion. To quantify these hepatocellular modifications by the pesticides, classification was carried out by dividing into five categories for a semi quantitative evaluation and, thus, rating microscopic sections relative to each other: 0, absent; 1, mild; 2, moderate; 3 severe. This categorization was made according to Capkin et al. (2010), Boran et al. (2010) and Atilgan et al. (2015).

Statistical analysis

The data were analysed by using SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA). Non-parametric Mann-Whitney U test was used to detect the significance of difference between the treated and control groups. $p \leq 0.05$ was considered as the level of significance.

Results and Discussion

The results obtained from the histopathological examination are given in Table 1. No histopathological changes

Table 1

Histopathological findings in the liver of Mus musculus exposed to Acm, Pro and mixture of them. Lesions were scored based on their severity (0: Absent; 1: Mild; 2: Moderate; 3: Severe)

Test substance	Concentrations (µg/ml)	Treatment period (h)	Vacuolar degeneration	Vascular dilatation	Sinusoidal dilatation	Treatment period (h)	Vacuolar degeneration	Vascular dilatation	Sinusoidal dilatation
Negative control	–-	24	0	0	0	48	0	0	0
Positive control	0.20	24	3*	3*	3*	48	3*	3*	3*
Acetamiprid	0.625	24	0	1	1*	48	1*	1	1*
	1.25	24	0	1	1*	48	0	1	1*
	2.50	24	1	2	1*	48	1	1	2*
Propineb	12.5	24	0	1	0	48	1	1*	1**
	25	24	2*	1	2*	48	0	1	0
	50	24	1*	1*	1**	48	0	1	0
Mixture of Pro and Acm	12.5 + 0.625	24	2*a	2	1*a	48	1*	1	1*
	25 + 1.25	24	2*a	2*	2*a	48	1**a	2*	2*b
	50 + 2.50	24	2*a	2*	1*	48	0	1*	1**b

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

a ($p \leq 0.05$); b ($p \leq 0.01$) each pesticide alone as compared to the mixture of Acm and Pro

were observed in livers of the negative control groups. Histopathological changes such as basophilic stippling, portal mononuclear cell infiltration, granulated cytoplasm, eosinophilic debris in liver of the pesticides-treated groups were observed. However, no significant differences were observed in these groups. The major histopathological changes in liver following an exposure to pesticides are showed in Figures 3–9.

When compared with the negative control, after both 24 and 48 h, all concentrations of Acm significantly induced formation of sinusoidal dilatation. Vacuolar degeneration was also significantly observed at the lowest concentration of Acm for 48 h.

The vacuolar degeneration in the hepatocytes and sinusoidal dilatation in the parenchyma were significantly observed in histopathological investigation of the liver sections

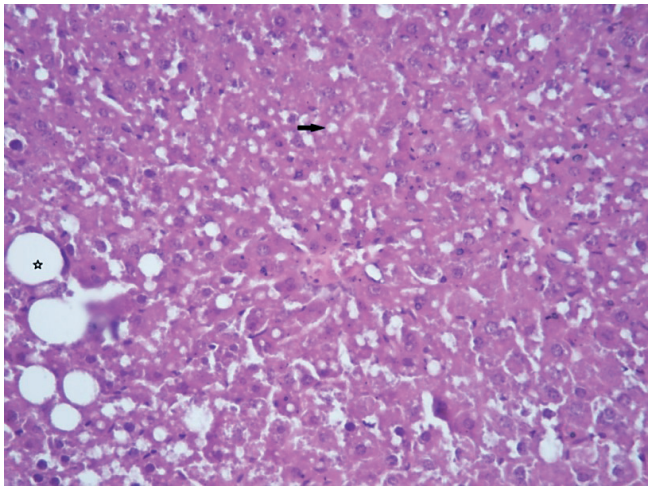

Fig. 3. Severe cell damage lesions were observed in the liver tissue after treatment with 25+1.25 µg/ml concentration of mixture of Acm and Pro for 24 h. Arrow: Vacuolar degeneration; Asterisk: Macrovesicular fatty change (H&E, 400x)

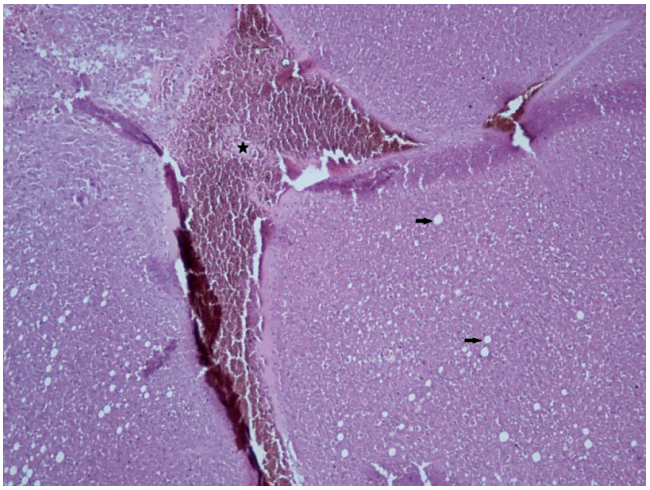

Fig. 5. Mild cell damage lesions were determined in the liver tissue showed after treatment with 12.5 µg/ml concentration of Pro for 48 h. Arrow: Macrovesicular fatty change; Asterisk: Vascular congestion (H&E, 400x)

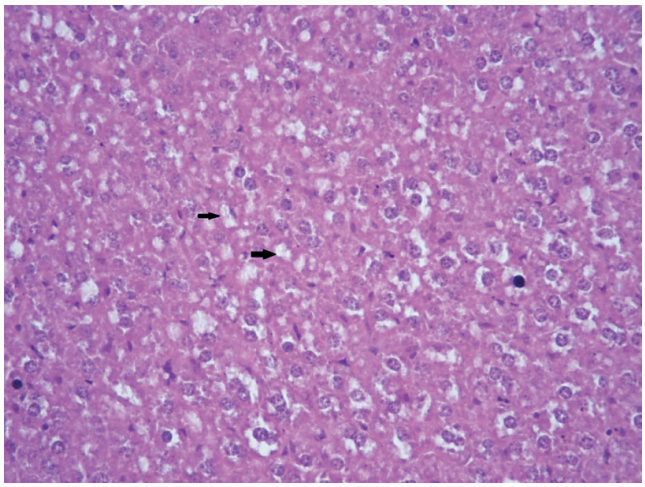

Fig. 4. Moderate vacuolar degeneration lesions were observed in the liver hepatocytes after treatment with 25 µg/ml concentration of Pro for 24 h (H&E, 400X)

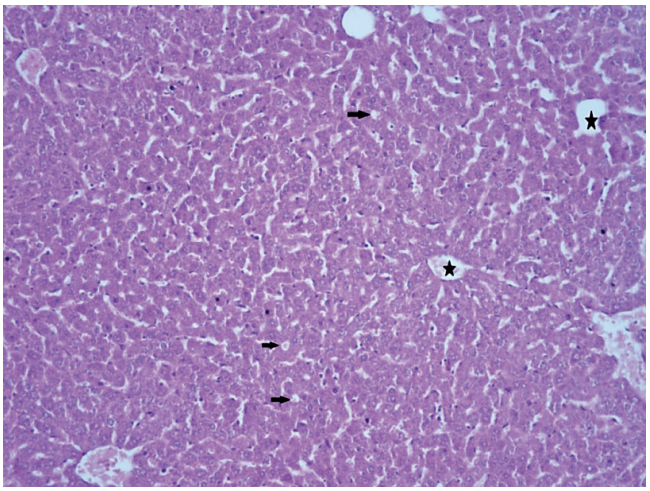

Fig. 6. The liver tissue showed severe cell damage lesions after treatment with 50+2.50 µg/ml concentration of mixture of Pro and Acm for 24 h. Arrow: Sinusoidal dilatation; Asterisk: Central vein (H&E, 400x)

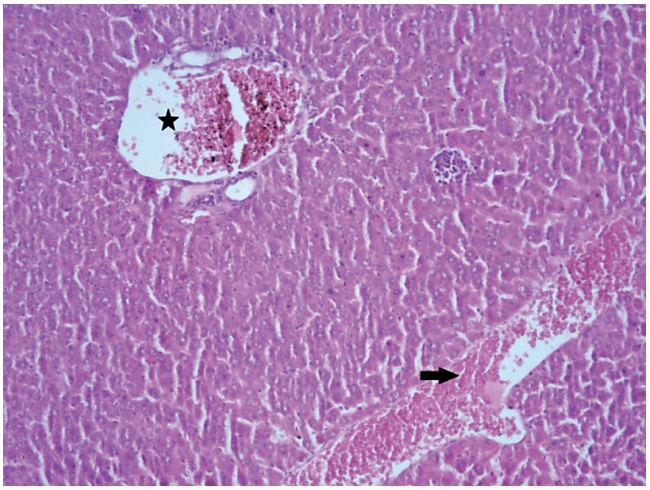

Fig. 7. The liver tissue observed moderate congestion at both portal vein (asterisk) and central vein (arrow); mild sinusoidal dilatation appearance after treatment with 25+1.25 µg/ml concentration of mixture of Pro and Acm for 48 h (H&E, 400x)

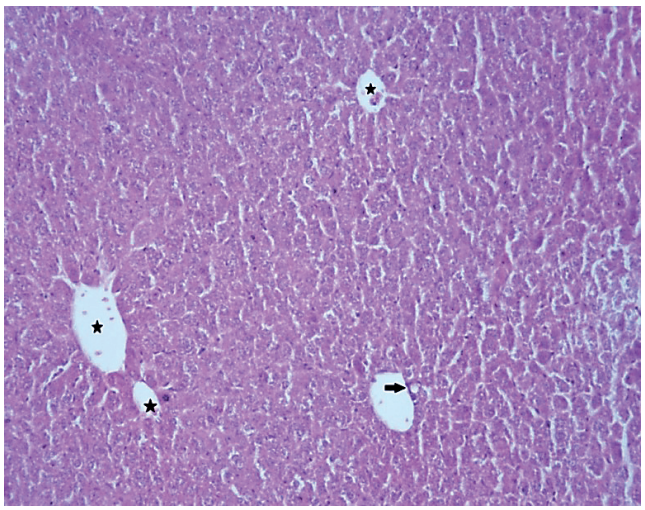

Fig. 8. Portal area (arrow) composed of portal vein and gall ductus and central veins (asterisk) were observed in the normal liver tissue after treatment with 0.625 µg/ml concentration of Acm for 24 h (H&E, 400x)

of mice treated with 25 and 50 µg/ml concentrations of Pro for 24 h when compared with the negative control. Moreover, vascular dilatation and sinusoidal dilatation were determined at the lowest concentration of Pro for 48 h.

It was also observed that the mixture of Acm and Pro significantly induced formation of vacuolar degeneration, vascular dilatation and sinusoidal dilatation in almost all of

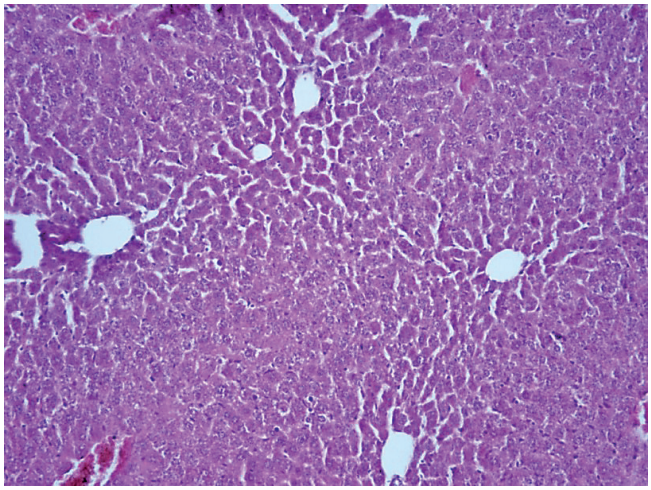

Fig. 9. The liver hepatocytes observed moderate sinusoidal irregularity appearance after treatment with 25 µg/ml concentration of Pro for 24 h (H&E, 400x)

the concentrations both 24 and 48 h when compared with the negative control.

The results of individually applied pesticides were also compared to results for their mixture at the same concentrations. According to the results, the mixture of Acm and Pro significantly produced synergistic effects (Table 1).

Occupational and environmental pesticide intoxication is a new threat to public health at present and there are many reports about occupational and environmental pesticide intoxication (Rambabu and Rao, 1994; Stefanelli et al., 2002; Rao et al., 2003; Costa et al., 2006; Reffstrup et al., 2010; Cavusoglu et al., 2011; Lee et al., 2012). Therefore, determining the effects of the chemicals, especially pesticides, is crucial because all living creatures exposed to these chemicals may be adversely affected.

The study presents the first *in vivo* evidence for the histopathological effects of separate and combined application of Acm and Pro on the liver of mice. There are a few studies on the histological effects of Acm, a neonicotinoid insecticide, on different tissues and organs in the different organisms. Păunescu et al., (2011) found that there were dilation of the marginal channel, hyperplasia of the epithelial cells and lifting of the lamellar epithelium in Prussian carp fish (*Carassius auratus gibelio* Bloch). It has been reported that Acm damaged seminiferous tubules and Leydig cells of mice (Zhang et al., 2011); renal corpuscles and tubules in kidney of mice (Zhang et al., 2012). However, previous studies also demonstrated that neonicotinoid insecticides such as imidacloprid, thiamethoxam and thiacloprid have caused impairment of the histopathological parameters in different

biological test systems. It was reported that imidacloprid induced histopathological alterations in liver of layer chickens (Kammon et al., 2010), liver tissue (Mohany et al., 2011; Toor et al., 2013) and brain tissue of rats (Soujanya et al., 2012), and malpighian tubules of honeybees (Rossi et al., 2013). It was also indicated that thiamethoxam caused histopathological changes in liver of rats (Shalaby et al., 2010), mice (Al-Sharqi et al., 2012), fish (Stoyanova et al., 2012), in hepatopancreas of terrestrial gastropod (Hamlet et al., 2012), and in plant roots (Martins et al., 2012). It was found that thiacloprid had moderate risk in *Gallus domesticus* (Goyal et al., 2010).

There are a few studies on the histological effects of Pro, a dithiocarbamate fungicide, on different tissues and organs in the different organisms. It has been revealed that Pro induced a variety of histopathological effects on the kidneys (Guven et al., 1998); livers, hearts and kidneys of fetus and female rats (Guven et al., 1999). Deveci et al., (1999) also reported that Pro and Maneb caused histopathological alterations in the livers of pregnant rats. Capkin et al., (2010) found that *Oncorhynchus mykiss* exposure to Pro produced necrosis, lipid infiltration, rise of sinusoidal space and rise of melanomacrophage centers. Furthermore, it was indicated that some dithiocarbamate fungicides such as carbosulfan, maneb, carbaryl have induced histopathological changes in fish tissue (Matos et al., 2007; Boran et al., 2010; Capkin et al., 2010; Gul et al., 2012).

There is no study on the histopathological effects of mixture of Acm and Pro on the liver of mice in the literature. However, it has been reported that many researchers found histopathological effects of mixture of pesticides on different tissues and organs in different organisms (Gyorkos et al., 1985; Strmac and Braunbeck, 2002; Xing et al., 2012; Wang et al., 2013).In this study, all concentrations of Acm and Pro mixture induced moderate histopathological lesions for 24 and 48 h.

Several mechanisms have been identified on liver cell injury (Grattagliano et al., 2002; Lee, 2003; Edinger and Thompson, 2004; Eren et al., 2004). One of them is mitochondrial dysfunction. It may occur in three different ways: Inhibition of fatty acid beta-oxidation, inhibition of respiratory enzymes or by a direct effect on mitochondrial DNA. Some chemicals inhibit both beta-oxidation and the functions of respiratory enzymes. Free fatty acids are not metabolized and lead to accumulation of lactate and reactive oxygen species. These radicals are damaged mitochondrial DNA. Consequently, some chemicals may cause hepatocellular necrosis, rapid disorganization of the hepatic architecture, breakdown of sinusoidal structures and pooling of blood in the liver through these mechanisms.

Conclusions

Liver is a target organ for detoxification and is prone to various disorders as a consequence of exposure to environmental pollutants. Histopathological alterations in tissue may be used as a rapid method to evaluate the toxic effects of chemicals in different tissues and organs (Bernet et al., 1999). According to the histopathological findings in this study, exposure to Acm, Pro and a mixture of them led to destructive effects on the liver tissue of mice. Both the results of this study and the findings of the previous studies showed that tissue alterations caused by chemicals in liver may result in severe functional problems and may lead to cell death in living creatures. However, to gain better insight into the mutagenicity and DNA damaging potential of Acm and Pro, further studies at molecular level should be conducted.

Acknowledgments

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