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The Effect of Acetamiprid Administration on Bcl-2 Immunoreactivity in the Liver

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ABSTRACT

This study aimed to show the effect of acetamiprid, a neonicotinoid insecticide, on B-cell lymphoma 2 (Bcl-2) gene expression, which plays an important role in apoptotic mechanisms in liver tissue. The study consisted of four groups in total, in which three doses of acetamiprid (5, 10, and 15 mg kg^{-1}) were administered, together with the negative group, in which no substance was administered. Liver tissues resected from mice sacrificed by cervical dislocation after 14 days of acetamiprid administration by gavage were fixed in a 10% formaldehyde solution for histological and immunohistochemical analyses and blocked in paraffin after routine tissue follow-up, and sections were stained with haematoxylin-eosin and immunostaining. Histological analysis revealed normal liver tissue in the control group; whereas, sinusoidal dilatation, vasodilatation, and necrosis and steatosis in the parenchyma were found in the acetamiprid-treated group at an increasing rate depending on the dose amount. The immunoreactivity of Bcl-2 in liver tissue was observed in the sinusoidal epithelium. Bcl-2 immunoreactivity was observed severely in the control and 5 mg kg^{-1} groups and moderately in the 10 mg kg^{-1} and 15 mg kg^{-1} acetamiprid-treated groups. Bcl-2 immunoreactivity was observed homogenously in the region from the central vein to the Kiernan's space. It was observed that acetamiprid used in the study showed a toxic effect on liver tissue, affected bcl-2 expression, an important biomarker in apoptotic pathways, and induced a dose-dependent decrease in bcl-2 immunoreactivity.

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Introduction

Pesticides are a chemical formulation used in agricultural activities and public health enterprises to combat fungi, insects, and weeds, as well as to eliminate insect-borne diseases. Since they are particularly effective against the organisms to which they are applied, their uncontrolled accumulation in nature tends to grow due to their rising use. Besides insects and other agricultural pests, the organisms on which it is primarily effective, unfortunately, also pose toxic effects on animals and humans at different levels. These effects vary depending on the dose and exposure level and the body's resistance (Toghan et al., 2022; Dogan et al., 2022; Deveci et al., 2021; Nur et al., 2021). Neonicotinoids are a class of pesticides consisting of nicotine-based compounds that are widely and successfully used against agricultural and household pests. Imidacloprid, acetamiprid, clothianidin, thiacloprid, thiamethoxam, dinotefuran, and nitenpyram are the most commonly used ones. Developed as a substitute for organophosphate and carbamate insecticides, they currently constitute the most extensively utilized class of pesticides (Çil et al., 2020; Bonmatin, 2015). These pesticides have stimulating properties akin to those of nicotine by attaching to acetylcholine receptors in the central nervous system. They bind tightly and irreversibly to nicotinic receptors found in insects. Therefore, toxic effects are greater in insects than in mammals and birds (Nur et al., 2023a; Kazuhiko et al., 2001). Acetamiprid is one of the second neonicotinoid group pesticides after imidacloprid. It is a white or very pale-yellow fine powder, crystalline, and odourless. Soluble in water, acetone, methanol, ethanol, dichloromethane, chloroform, acetonitrile, and tetrahydrofuran. The restriction of the use of nitro group-containing neonicotinoids in the European Union (2013) due to their acute toxic effects on pollinators has led to a rise in the use of cyano group-containing acetamiprid. While most of neonicotinoids are systemically effective and used in seed coating, acetamiprid is usually applied by spraying on leaves (Camp et al., 2020). Unfortunately, except for its use in pest control, it accumulates in vital organs in other organisms and 60-75% of acetamiprid is remove as metabolites in faeces and urine. Few studies have demonstrated the

harmful effects of acetamiprid. However, the main signs of exposure to acetamiprid, particularly in people, are headache, nausea and vomiting (Chakroun et al., 2016). Apoptosis, a response to cellular stress or developmental cues, is assessed to be an important biological process. Disruptions in the apoptosis process can lead to pathological lesions, cancer development in more advanced stages, and even negative responses to conventional chemotherapy applications. The B-cell lymphoma 2 family exhibits activity as both an apoptosis inducer and suppressor. Especially in recent years, researchers have focused on the apoptotic mechanism, tumor formation and its effects on pathways in cytotoxic treatments (Qian et al., 2022). The BCL-2 family, an important protein family related to programmed cell death (apoptosis), contains both inhibitory (anti-apoptotic) and regulators (pro-apoptotic) blocking the inhibitory effects (Ploumaki et al., 2023; Chalazonitis et al., 2012).

The aim of this research is to reveal the effect of acetamiprid-one of the neonicotinoid group pesticides that can induce immune, nervous, respiratory, and reproductive system disorders, as well as liver and kidney tumours on Bcl-2 gene expression, which plays a role in apoptotic mechanism in liver tissue.

Materials and Method

Male Mus musculus var. albinos mice, weighing 25–30 g and roughly 8 weeks old, were used in this investigation. Appropriate feed and tap water were used to feed the mice. Mice housed under ambient conditions including 21±2°C temperature, 50% humidity, twelve hours of daylight and twelve hours of darkness. Test animals were housed in polycarbonate-derived lattices that could be autoclaved at 121°C. Substance dose adjustments were prepared daily according to the weight of the animals and administered orally. Mice were randomly distributed to groups created in the study. The animals were given anesthesia and killed by cervical dislocation at the conclusion of the study protocol. The liver was then weighed on a precision balance, normalised to body weight, and used in statistical analyses. All experimental procedures in the study were approved by the Kafkas University Animal Experiments Local Ethics Committee (17/03/2017-093).

Group I: (Negative Control Group, n: 10): The mice in this group, designated oral gavage of distilled water was given to as the negative control group.

Group II: (5 mg/kg Acetamiprid group, n:10): For 14 days, the mice in this group received oral gavage with 5 mg/kg of acetamiprid.

Group III: (10 mg/kg Acetamiprid group, n:10): For 14 days, the mice in this group received oral gavage with 10 mg/kg of acetamiprid.

Group IV: (15 mg/kg Acetamiprid group, n:10): For 14 days, the mice in this group received oral gavage with 15 mg/kg of acetamiprid.

Organosomatic Index

The liver weight information was used to compute the organosomatic index (OI):

$$OI = (\text{tissue weight}/\text{body weight}) \times 100$$

Histological Analysis

For detection, tissue samples from mice that were killed via cervical dislocation were preserved in a 10% phosphate-buffered formalin solution. Following a thorough washing under running tap water, the fixed tissue samples were subjected to a series of alcohol grades, representing 60%, 70%, 80%, 90%, 96%, and 100% dehydration, respectively. The samples were placed in xylene to render the tissues translucent. Once transparent, they were embedded in paraffin blocks and incubated in liquid paraffin for three hours at 60°C in an oven. Using a microtome, slices 5 µm thick were cut from paraffin blocks on slides covered with chrome alum gelatin (CAG). Hematoxylin-Eosin (HE) was used to stain the slices so they could be examined under a light microscope. (Suvarna and Layton, 2019).

Immunohistochemical Analysis

Anti-Bcl-2 (B-cell lymphoma 2, ab59348) primary antibody was applied at a ratio of 1/100 diluted to 5 µm sections prepared from paraffin blocks for 1 hour at room temperature in a humid medium in order to assess Bcl-2 positivity in liver tissue. The only treatment given to the sections in the negative control group was phosphate buffer solution (PBS). After the primary antibodies were incubated, one of the indirect methods, the Streptavidin-biotin peroxidase technique, was used. Next, 3-Amino-9-Ethylcarbazole (AEC) was added as a chromogen (Shu et al., 1988). When immunoreactivity was observed as a result of controlled examinations under the light microscope, the reaction was terminated with distilled water. Mayer haematoxylin was used for counterstaining. Photographs were taken under a light microscope, taking care to randomisation of the site selection, and the distribution and intensity of immunoreaction were investigated semi-quantitatively according to an immunoreactive score (Seidal et al., 2001; Zhu, 1989; Gelen et al., 2017). Accordingly, a score of 0 was assigned if there were no positive cells; a score of 1 was assigned if there were less than 1/100 positively stained cells; a score of 2 was assigned if the rate was between 1/100 and 1/10; a score of 3 was assigned if the rate was between 1/10 and 1/3; a score of 4 was assigned if the rate was between 1/3 and 2/3; and a score of 5 was assigned if the rate was >2/3. Following that, a score was calculated to represent the mean density of positive cells. As a result, the following scores were assigned: 0 for no staining, 1+ for weak immunoreactivity, 2+ for moderate immunoreactivity, and 3+ for strong (increased) staining.

Statistical Analysis

The SPSS 22.0 program was used to conduct the statistical analyses (SPSS Inc. Chicago, Illinois, USA). During the data analysis process, one of the multiple comparison tests, the Tukey HSD test, and one-way analysis of variance were used to compare three or more groups. $P<0.05$ and $P<0.01$ values for the tests' probability of error were accepted.

Results

Findings on the Liver Weight

By the conclusion of the experimental protocol, liver tissues from sacrificed animals were obtained and weighed, and the changes in weight are presented in Table 1. The data analysis showed that exposure to acetamiprid resulted in a loss in liver tissue weight. The 5 mg kg^{-1} acetamiprid dose did not appear to cause a statistically significant loss in liver tissue weight when in contrast to the control group (P>0.05). The reduction in liver weight was higher in the acetamiprid groups treated with 10 mg kg^{-1} and 15 mg kg^{-1}. When compared to the control group, this amount of decrease is statistically significant (P<0.01). Compared to the control group, the group that received the highest dose of acetamiprid administration experienced a 9.23% weight loss.

Table 1. Acetamiprid's effects on the groups' liver organ weights (g).

Parameter	Groups (Mean±SD) (n=10)				P
	Control (n=10)	5 mg kg^{-1} acetamiprid	10 mg kg^{-1} acetamiprid	15 mg kg^{-1} acetamiprid	
(Liver weight/Live weight)×100	0.65±0.02[a]	0.63±0.01[a]	0.6±0.02[b]	0.59±0.01[b]	*

*P<0.01: Statistically significant difference, [a,b]: Values with different letters indicate significant differences, n: number of animals in the group, SD: Standard deviation

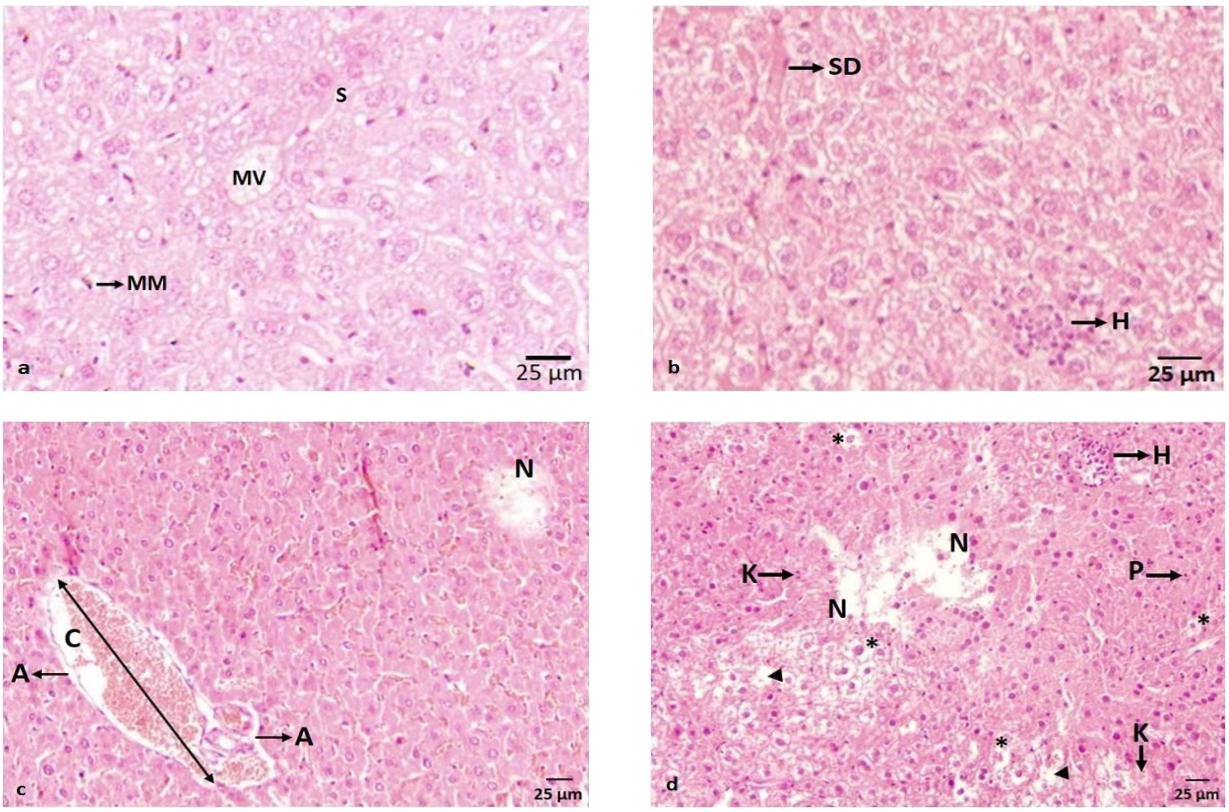

Figure 1. a. Liver histology of the negative control group, b. Liver histology of 5 mg/kg acetamiprid-treated group, c. Liver histology of 10 mg/kg acetamiprid-treated group, d. Liver histology of 15 mg/kg acetamiprid-treated group, central vein (CV), sinusoid (S), melanomacrophage centre (MC), sinusoidal dilatation (SD), haemorrhage (H), vasodilation (C), necrosis (N), steatosis (*), karyorrhexis (K), pyknosis (P), atrophy (arrowhead), H&E, bar: 25 µm.

Histopathological Findings

The liver cells located around the central vein and erythrocytes located in the sinusoids can be distinguished in the liver histology of the negative control group samples in which no treatment was administered. A small amount of melanomacrophages was observed in the liver parenchyma. The liver histology of the 5 mg/kg acetamiprid-treated group shows dilatation of the sinusoids as well as mild haemorrhage in the parenchyma. The liver histology of the 10 mg/kg acetamiprid-treated group showed marked dilatation of the portal vein and separation between the parenchyma and the vein. There is localised necrosis in the parenchyma. The liver histology of the 15 mg/kg acetamiprid-treated group showed marked haemorrhage and necrosis in the parenchyma. Fat deposition findings are typically observed in the parenchyma. Consequently, hepatocytes lost their normal shape, and the nuclei having karyorrhexis and pyknosis structure were pushed towards the periphery of the cells. Among them, there are also atrophic hepatocytes that have lost their nuclei (Figure 1 a, b, c, d).

Table 2. Tissue alterations and histopathologic lesion ratings in Mus musculus var. albinos liver tissue. (Frequency ratings have been adapted from Sakat et al., (2019)).

Tissues	Lesions	Control Group	Acetamiprid dose groups		
			5 mg kg^{-1}	10 mg kg^{-1}	15 mg kg^{-1}
Liver	Hydropic and vacuolar degeneration of hepatocytes	0	0	+	++
	Irregularity in remark cords	0	0	+	+++
	Congestion	0	+	++	++
	Vasodilation	0	+	++	++

0 No anomaly, +: Low frequency of abnormality, ++ Moderate frequency of abnormality, +++: High frequency of abnormality

Figure 1. a. Liver histology of the negative control group, b. Liver histology of 5 mg/kg acetamiprid-treated group, c. Liver histology of 10 mg/kg acetamiprid-treated group, d. Liver histology of 15 mg/kg acetamiprid-treated group, central vein (CV), sinusoid (S), melanomacrophage centre (MC), sinusoidal dilatation (SD), haemorrhage (H), vasodilation (C), necrosis (N), steatosis (*), karyorrhexis (K), pyknosis (P), atrophy (arrowhead), H&E, bar: 25 µm.

Immunohistochemical Findings

The liver's sinusoidal epithelium exhibited bcl-2 immunoreactivity when liver tissue was immunohistochemically analyzed. Upon group analysis, the control group's bcl-2 immunoreactivity was found to be quite high (Figure 2a, b, c, d) and 5 mg kg-1 acetamiprid groups (Figure 2e, f, g), and moderate in the 10 mg kg-1 (Figure 2i, j) and 15 mg kg-1 (Figure 2k, m) acetamiprid groups. The bcl-2 immunoreactivity in the liver tissue was observed homogeneously in the region from the central vein to Kiernan's spaces.

Figure 2. Bcl-2 immunoreactivity in rat liver tissue. Immunoreactivity was severe in the control and 5 mg kg^{-1} acetamiprid groups and moderate in the 10 mg kg^{-1} and 15 mg kg^{-1} acetamiprid groups. Control group (a, b, c, d), 5 mg kg^{-1} acetamiprid group (e, f, g), 10 mg kg^{-1} acetamiprid group (ı, j), 15 mg kg^{-1} acetamiprid group (k, m). CV: vena centralis, Kiernan's spaces: rectangular area.

Discussion

The class of insecticides that is most frequently used globally is made up of highly water-soluble organic pesticides called neonicotinoids (El-Garawani et al., 2022). Neonicotinoid toxicity has been proven by numerous studies in bees and insects. However, its effects on mammalian tissues have not yet been sufficiently investigated (Burke et al., 2009). Acetamiprid is a neonicotinoid insecticide extensively used for agricultural, domestic, and public health management activities around the globe (Phogat et al., 2023). Its widespread use and water-soluble nature exert significant risk to the environment and life when not used according to label directions (Gaweł et al., 2019). Residues of acetamiprid and its metabolites have been frequently detected in soil (Bonmatin et al., 2021), water (Zoumenou et al., 2019), food (Craddock et al. 2019), fruits (Wu et al., 2012), and agricultural products (Gupta et al., 2005; Pramanik et al., 2006), thereby making the non-target organisms more susceptible due to exposure. The liver and kidney were observed to be the primary target organs affected by acetamiprid, which has also been observed to have genotoxic, neurotoxic, and cytotoxic effects (Önen et al., 2018).

The first analysis of our study's data shows that applying acetamiprid reduces the weight of liver tissue. A study using cypermethrin application revealed that kidney tissue had gained weight, whereas liver tissue had lost volume and weight when the studies that other researchers had done with organ weight were evaluated (Sangha et al., 2011). It was observed that the use of arsenic and malathion separately and together led to a loss in body weight and liver weight but a rise in weight in brain tissue (Naraharisetti et al., 2009). Another practice found that fipronil caused liver and kidney weight gain (Swelam et al., 2017). The study conducted by Nur et al,. reported in their study that while the weight gain in kidney tissue was statistically insignificant at the lowest dose of 5 mg kg^{-1} in acetamiprid-treated mice compared to the control group, the gain in kidney tissue was significant at doses of 10 and 15 mg kg^{-1} in contrast to the control group (Nur et al., 2022).

Following acetamiprid administration, sinusoidal or venal dilation, locally separation between the deformed vena circumference and parenchyma, haemorrhage, steatosis, the presence of karyorrhectic, pyknotic, and atrophic nuclei in hepatocytes, and necrosis findings were observed in the liver tissue, and the lesions appeared to further enlarge as the dose increased. Acetamiprid and imidacloprid have been reported to cause histopathologic lesions on the gills, liver, and muscles of *Oreochromis niloticus* and to exert genotoxic effects by forming nuclear abnormalities and micronuclei in erythrocytes (El-Garawani et al., 2022). It has been reported that acetamiprid causes inflammatory cell infiltration, congestion and necrosis in the rat liver, while it causes tissue damage such as gliosis and hyperemia necrosis in the brain tissue. Especially due to the increase in oxidative stress, an increase in oxidant indicator levels and a decrease in antioxidant enzyme levels have been shown in serum biomarkers (Khovarnagh and Seyedalipour, 2021). Similar to the literature, these hepatotoxic effects suggest that the liver is more susceptible to the cytotoxic effects of neonicotinoid group pesticides due to the role of the liver as a detoxification centre and as the tissue where foreign substances such as toxins and chemicals penetrating the body are detoxified (Kammon et al., 2010; Badgujar et al., 2013; Soujanya et al., 2013; Arfat et al., 2014; Kumar et al., 2014). As a result of exposure of mice to acetamiprid and propineb, severe vacuolar degeneration of hepatocytes of liver tissue and sinusoidal dilatation of the liver parenchyma were found (Rasgele et al., 2015).

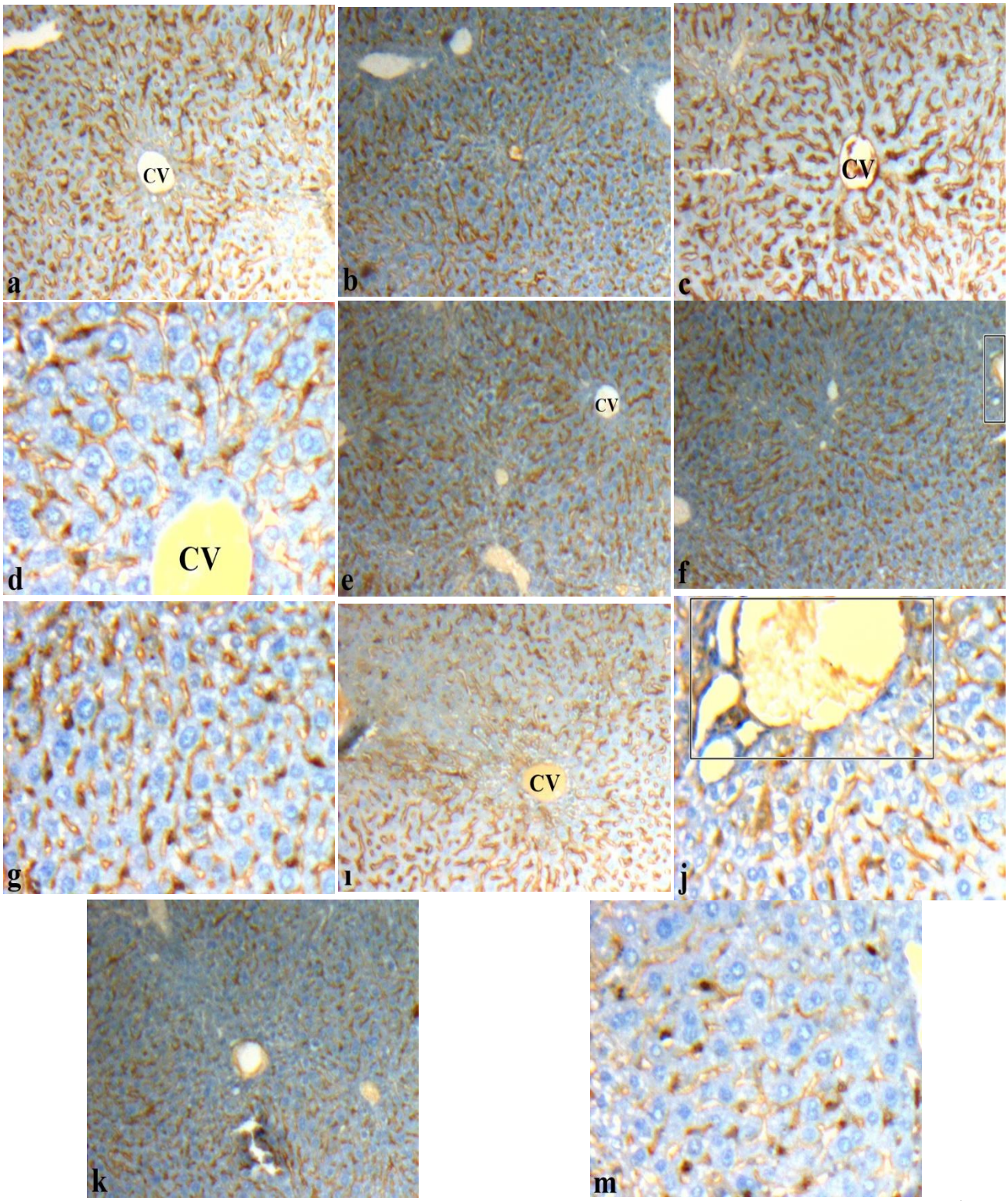

Figure 2. Bcl-2 immunoreactivity in rat liver tissue. Immunoreactivity was severe in the control and 5 mg kg^{-1} acetamiprid groups and moderate in the 10 mg kg^{-1} and 15 mg kg^{-1} acetamiprid groups. Control group (a, b, c, d), 5 mg kg^{-1} acetamiprid group (e, f, g), 10 mg kg^{-1} acetamiprid group (ı, j), 15 mg kg^{-1} acetamiprid group (k, m). CV: vena centralis, Kiernan's spaces: rectangular area.

Additionally, a study on the effects of acetamiprid on the kidneys—one of the organs most impacted by pesticides-showed that an increase in the dose of the medication caused glomerular lobulation, glomerular atrophy, and degeneration in the proximal and distal tubules. In groups to which caffeic acid phenethyl ester was administered as a protector against this damage, a regression was observed in pathologic lesions (Nur et al., 2023b). In their investigation, Karaca et al. used varying acetamiprid dosages and found that the liver had more pathological damage than the kidneys (Karaca et al., 2019). A study in which acetamiprid was administered to rats showed adiposity, leukocytic infiltration, and haemorrhage in liver tissue, while tubular atrophy, occluded blood vessels, dilatation, and dense eosinophilic cytoplasm were observed in kidney tissue (Toghan et al., 2022). Results

from the present study and other studies suggest that acetamiprid has hepatotoxic effects on liver tissue.

BCL-2 homology (BH) domains vary amongst BCL-2 family members and can be used to distinguish them through expression analysis. The most important members of this family with anti-apoptotic properties are BCL-2 and BCL-XL, while important members with pro-apoptotic effects are Bax, Bak, and BCL- $_{Xs}$ (Ploumaki et al., 2023; Chalazonitis et al., 2012). This protein family is responsible for mitochondrial outer membrane permeability and as a result, caspase cascade activation (Singh et al., 2019). Gür et al., examined the effect of quercetin in obesity-induced rats. According to their findings, oxidative stress induced by experimental obesity increased Bax immune reactivity while decreasing Bcl-2 immunoreactivity. An increase in Bcl-2 gene expression was observed in the obesity group administered Qurcetin (Gür et al., 2022). While p53 immunoreactivity increased in the liver in hepatotoxicity induced by silver nanoparticles, Bcl-2 immunoreactivity decreased. According to a study, when eugenol was given to the group exposed to silver nanoparticles instead of just silver nanoparticles, p53 immunoreactivity dropped and Bcl-2 expression rose (Yousef et al., 2022). It was reported that thiamethoxam, one of the neonicotinoids, induced pathological lesions in the uterus and ovaries of rats and increased caspase-3 immunoreactivity in the uterus and ovaries compared to the control group. Furthermore, it was reported that Bcl-2 levels, one of the markers associated with apoptosis, were down-regulated, the Bax/bcl-2 ratio rose by up to 52% in contrast to the control group, and p53 expression was up-regulated (El-Din et al., 2023). A study by Nur et al. in which they examined the immunoreactivity of Bcl-2 and p53 apoptotic markers showed an increased expression of p53—one of the cell death receptors parallel with the pathological changes in the kidneys resulting from acetamiprid-induced nephrotoxicity and a decrease in anti-apoptotic Bcl-2 expression due to suppression (Nur et al., 2022).

Acetamiprid one of the neonicotinoids, which is regarded as safer than other pesticide groups nowadays is used to eliminate insects, especially in the agricultural industry, but it has been recognised that it may be a potential threat to living organisms other than its intended use. Acetamiprid induces stress on the vital pathways of the cell by causing the increase of reactive oxygen species. Thus, it was discovered that the amount of acetamiprid given affected the immunoreactivity of bcl-2, one of the apoptotic markers. Administration of acetamiprid was found to alter the transcription of the apoptosis-related Bcl-2 gene. Acetamiprid should be used in recommended doses in agricultural activities due to its intended use. However, it can enter the food chain due to contamination with aquatic ecosystems and pesticide residues in agricultural products. It accumulates at an increasing rate in the tissues of living things through the food chain, which triggers damage to cells and tissues. For these reasons, we think that combating agricultural pests through their natural enemies should be encouraged within the scope of popularizing green agricultural practices in the future. Ensuring that the use of this pesticide is minimized is very important for clean and sustainable agriculture in the future. Results of the study indicated that although acetamiprid is regarded as safer than other pesticide groups, acetamiprid has negative effects on other organisms besides insects, the target organisms, and acts in favour of the oxidant mechanism on the oxidant-antioxidant system, which serves a crucial function for the organism's survival and, as such, is toxic to other living things.

Conflict of Interest/Competing Interests: Not available.

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