TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2022) 46: 795-802

doi:10.55730/1300-0128.4255

Pathological and immunohistochemical changes in the liver of monocrotaline-treated rats

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Received: 05.09.2022 • **Accepted/Published Online:** 05.11.2022 • **Final Version:** 08.12.2022

Abstract: Pathological and immunohistochemical changes in livers were investigated in monocrotaline-treated rats. Eighty adult-male Wistar rats were distributed in 5 groups, each containing 16 animals according to monocrotaline dose (Group I: 50 mg/kg, Group II: 100 mg/kg, Group III: 150 mg/kg, and Group IV: 200 mg/kg). The control group was administered with physiological saline. Half of the animals in each group were euthanized at the 18th hour while the remaining half at the 6th week postinoculation. At the end of the 18th hour, the most prominent lesion was hepatocytic apoptosis which was dose-dependent. Apoptotic cells were mostly located in periportal and mid-zonal areas. The TUNEL staining was consistent with morphological findings. Mild to moderate sinusoidal congestion, vacuolar and fatty degeneration were present in all the experimental groups. On the other hand, no difference was detected between the experimental and control groups in proliferating cell antigen nuclear (PCNA) staining, whereas Bax/Bcl-2 staining was present in all the experimental groups with a dose-dependent manner. At the end of the 6th week, hepatic megalocytosis, bile duct proliferation, postnecrotic fibrosis were the main findings. The severity of the lesions was dose-dependent, and few intranuclear inclusion bodies were also detected at the highest dose. At this period, hepatocytes were slightly stained by Bax and Bcl-2, whereas PCNA and TUNEL staining were intensive. In conclusion, monocrotaline showed different effects on the liver depending on dose and duration of the exposure, and the use of doses below 100 mg/kg may be recommended for long-term monocrotalin toxicity studies.

Key words: Immunhistochemistry, liver, monocrotaline, pathology, rat

1. Introduction

Pyrrolizidine alkaloids (PAs) are widely distributed herbal toxins in nature [1-3]. There are about 660 PAs with different structure determined in 6000 plant species of 13 plant families [4-8]. Roughly, 3.0% of the world's flowering plants contain the PAs [2,9-12].

The liver is the primary target organ in PA toxication [2,13]. Pyrrolic metabolites constitute the alkylating reaction with the structural elements of tissues [14]. They form covalent bonds with cellular structures such as DNA, protein, amino acid, and glutathione [2,9,13]. Besides the hepatotoxic effects, PAs have carcinogenetic [11,15-17], antimitotic, and immunosuppressive effects [18].

The antimitotic effects are attributed to binding of the pyrrolic metabolites to proteins or nucleic acids [3,11]. Pyrroles cause degeneration and necrosis in cells through binding proteins of cytoplasm and the thiol groups of nucleic acids [9,11]. Due to attachment of the metabolites to the specific target regions on DNA, G2 and M phases of mitosis do not occur; however, G1 and S phases are not affected. Hepatocytes return to G1 phase again, neglecting G2 or M, and protein synthesis continues in the cell [2]. As a result of antimitotic effects, megalocytic hepatocytes are formed in chronic toxication [1,19-21]. Mutagenic and teratogenic effects also occur when the pyrollic metabolites bind to the DNA helix [13,22]. The carcinogenic effect of PAs is caused by damage of the P53 gene in the DNA [22].

Monocrotaline is a pyrrolizidine alkaloid plant toxin that causes hepatotoxic, pneumotoxic, genotoxic, and nephrotoxic effects in human and animals [5,6,8,10,23].

Although there are many studies on pneumotoxic and genotoxic effects of monocrotaline, its time course and dose-related hepatotoxic effects have been examined in a limited number of studies [6].

This study was performed to investigate the time course and dose-related pathological findings induced by monocrotaline in Wistar rat liver.

2. Materials and methods

2.1. Animals

The present study was approved by the Ethics Commission of Fırat University in the convention on 05.02.2014 with the decision number 38.

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Eighty adult-male Wistar rats were used in the study which was performed at Fırat University Experimental Animal Research Center (FUDAM). The animals were divided into 5 groups including 4 experimental and one control group. Each group consisted of 16 animals. During the experiment, the rats were fed with standard rat diet and water as ad libitum.

2.2. Monocrotaline

Monocrotaline in solid form (Crotaline, Sigma, Catalog No. 315-22-0) was diluted with distilled water and prepared 10% solution (100 mg monocrotaline in 1 mL). The pH of the solution was regulated to 7.20 with addition of sodium bicarbonate solution.

2.3. Experimental toxicology

At the beginning of the experiment, the rats were weighted and recorded. When constituting the groups, the average live weights of the rats in each group were paid attention to be close to each other. Monocrotaline solution was injected intraperitonally (i.p.) at the dose of 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg to the Groups I, II, III, and IV, respectively. The rats in the control group were injected with a single dose of 1 mL serum physiological i.p.

2.4. Histopathological method

Systemic necropsy was performed in rats that died during the experiment period and in those that were euthanized at the end of experiment (18th hour and 6 weeks). Liver samples were collected and fixed in 10% buffered formalin. Paraffin blocks were cut at 3-5 μm thickness and the sections were stained with hematoxylin-eosin (HE) for routine histopathological examinations. Histopathological findings were scored between 0 and 5 according to the severity of the lesions. According to this score, 0 was graded as no lesion, 1 to 2 as mild, 3 to 4 as moderate, and 5 as severe.

2.5. Immunohistochemical method

The liver sections were stained by avidin-biotin-complex (ABC; DAKO, Carpinteria, CA, USA) method with proliferating cell nuclear antigen (PCNA, mouse, monoclonal, 1:200, Santa Cruz), B-cell lymphoma (Bcl-2, rabbit, polyclonal, 1:400, Santa Cruz), B-cell lymphoma associated X protein (Bax, rabbit, polyclonal, 1:400, Santa Cruz) primary antibodies to determine the rate of mitotic activity. ABC staining method was used according to the manufacturer's protocol. Briefly, the sections were dewaxed and rehydrated, and washed three times in phosphate buffered saline (PBS) solution. For antigen retrieval, sections were placed in coplin jar containing citrate buffer solution (pH 6.0) and heated in microwave oven for 15 min at the highest power setting (distilled water was added every 5 min). The jar was then removed from the oven and allowed to cool slowly at room temperature. To block endogenous peroxidase, the sections were treated with 3.0% hydrogen peroxide in methanol for 5 min. Following the incubation with blocking solution, the sections were treated with primary antibodies (PCNA, Bcl-2, Bax) at 4.0 °C in a refrigerator overnight. After treatment with biotinylated secondary antibody for 30 min, the slides were incubated with strepavidin peroxidase for 30 min. Except for incubation with blocking solution, slides were washed with PBS 3 times for 5 min during all incubations with reagent intervals. Following visualization of the positive antigen-antibody reaction by incubating with AEC chromogen, the slides were slightly counterstained with Mayer hematoxylin.

2.6. TUNEL method

The liver sections were stained by using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) method for detection of apoptosis. The TUNEL staining was done using an Apoptag Plus Kit (Millipore, Canada USA) according to the manufacturer's protocol. Mouse thyroid tissue which was fixed in formalin and embedded in paraffin was used as positive control. A previously experimentally degenerated mouse retina was used as a control.

2.7. Calculation of live weight and organ

The rats were weighed before the trial and then once a week from the start of the experiment during 6 weeks. The Kruskal–Wallis variance analysis was used to determine the statistical difference between the groups. The Mann–Whitney U test was used to measure the difference between the groups. Necropsy was performed on the animals that died during the experiment and on those that were euthanized at the end of the experiment, and organ weights were determined.

3. Results

3.1. Live weights

Live weights of animals in the experimental groups were lower than in the control group; however, there was no significant difference between the experimental groups.

3.2. Clinical findings

In the high-dose-administered groups (Groups III and IV); weakness, unresponsiveness to external stimuli, anorexia, reluctance to move, and weight loss were noted from the first week to the end of the study when compared to the control group. The deaths in the experimental groups were recorded as two rats in Group I (on the 37th and 39th days), six rats in Group II (one each on the 29th and 30th days and four on the 40th day), seven rats in Group III (three on the 4th day, two on the 6th day, and one each on the 39th and 40th days), and seven rats in Group IV (three on the 7th day, and two each on the 8th and 38th days). No death occurred in the Control group. At the 18th hour of postinoculation, 8 animals from each

group were euthanized and necropsied. In the long-term trial period, animals that died after the 35th day and those that remained alive until the end of the trial were evaluated within the scope of the 6th week.

3.3. Gross findings

In the necropsy performed at the end of the 18th hour, the macroscopic findings in Groups I, II, and III were milder than those in Group IV. In Group II, a moderate icterus was present on the serosal surfaces in the abdominal cavity in addition to like honeycomb pethechiae on the liver lobes of an animal that died on the 29th day. There was a coconut-like appearance in visceral surface, milier shape and pale white foci in the livers of the animals that died on the 7th and 8th days in Groups III and IV. In Group IV, 2–4 mL of light yellow and clear ascites fluid in abdominal cavity of 3 animals necropsied at the end of 18th hour were noted.

3.4. Liver weights

A statistically significant difference was detected between the experimental and control groups in terms of liver weights at the 18th hour of the study, whereas the difference among the experimental groups was not significant (Tables 1 and 2). At the end of the 6th week, a statistically significant difference was present between Group I, Group II, Group III, Group IV, and Group V (control).

3.5. Microscopic findings

3.5.1 Microscopic findings in the liver of rats euthanized at the end of the 18th hour

Semiquantitative evaluation of histopathological findings in liver at the end of 18th hour was summarized in Table 3. In all experimental groups, vacuolar degeneration and Kupffer cell activation were observed at varying degrees of severity in addition to focal necrosis foci. Histopathological changes were more severe in the high-dose groups. As seen in Table 3, total liver damage in Group IV occurred 8 times more than in Group I. Similarly, apoptosis which was detected in all experimental groups with increased proportion depending on the dose was recorded as the most important microscopic finding detected at the end of the 18th hour. While 1-2 apoptotic nuclei were found in each microscope area in the low dose groups (Groups I and II), clusters of apoptotic particles were detected in the regions in the high dose groups (Groups III and IV) (Figure 1A). These cells were dark eosinophilic and round cytoplasm, nuclei shrunk and separated from the surrounding cells. Periportal lymphocytic infiltrations and mild-to-moderate multifocal sinusoidal congestion were found in the animals in the high dose groups (Groups III and IV). In the experimental groups, megalocytosis was not observed, but only sporadic karyomegalic hepatocytes and numerous dual nucleated liver cells were found in the high-dose groups (Figure 1B).

3.5.2. Microscopic changes in the liver of rats euthanized at the end of the 6th week

Semiquantitative evaluation of histopathological findings in liver at the end of the 6th week was summarized in Table 4. At the end of this period, severe diffuse megalocytosis characterized with the increased volume of cell nuclei and cytoplasm was the most important finding detected in Groups III and IV (Figure 1C). The diffuse bile duct proliferation was observed in all experimental groups, especially in Groups III and IV (Figure 1D). The postnecrotic fibrosis and mild-moderate capsular fibrosis were observed to accompany the abovementioned lesion in Groups III and IV. Besides mononuclear cell infiltrations

Table 1. Mean liver weights at the end of the 18th hour according to the groups (g).

Tissue	Group I X ± SX	Group II X ± SX	Group III X ± SX	Group IV X ± SX	Group V X ± SX	p
Liver	7.659 ± 0.757^{b}	7.255 ± 1.234^{b}	7.97 ± 0.788^{b}	7.263 ± 1.330^{b}	10.793 ± 0.739^{a}	*

*: $p < 0.05$
Different superscripts (^{a,b}) in the same row indicate significant difference ($p < 0.05$).

Table 2. Mean liver weights of groups at the end of the 6th week (g).

Tissue	Group I X ± SX	Group II X ± SX	Group III X ± SX	Group IV X ± SX	Group V X ± SX	p
Liver	10.016 ± 1.177^{bc}	7.474 ± 1.884^{b}	5.961 ± 1.087^{a}	5.614 ± 0.276^{a}	10.836 ± 0.884^{c}	*

*: $p < 0.05$
Different superscripts (^{a,b,c}) in the same row indicate significant difference ($p < 0.05$).

Table 3. Semiquantitative evaluation of histopathological findings in livers at the end of the 18th hour.

	HD	FD	SC	N	ICI	TS
Group I	1	0	1	0	0	2
Group II	1	0	1	3	0	5
Group III	3	0	2	3	1	9
Group IV	4	1	4	5	2	16
Group V	0	0	0	0	0	0

HD: Hydropic degeneration, FD: Fatty degeneration, SC: Sinusoidal congestion, N: Necrosis ICI: Inflammatory cell infiltration, TS: Total score.

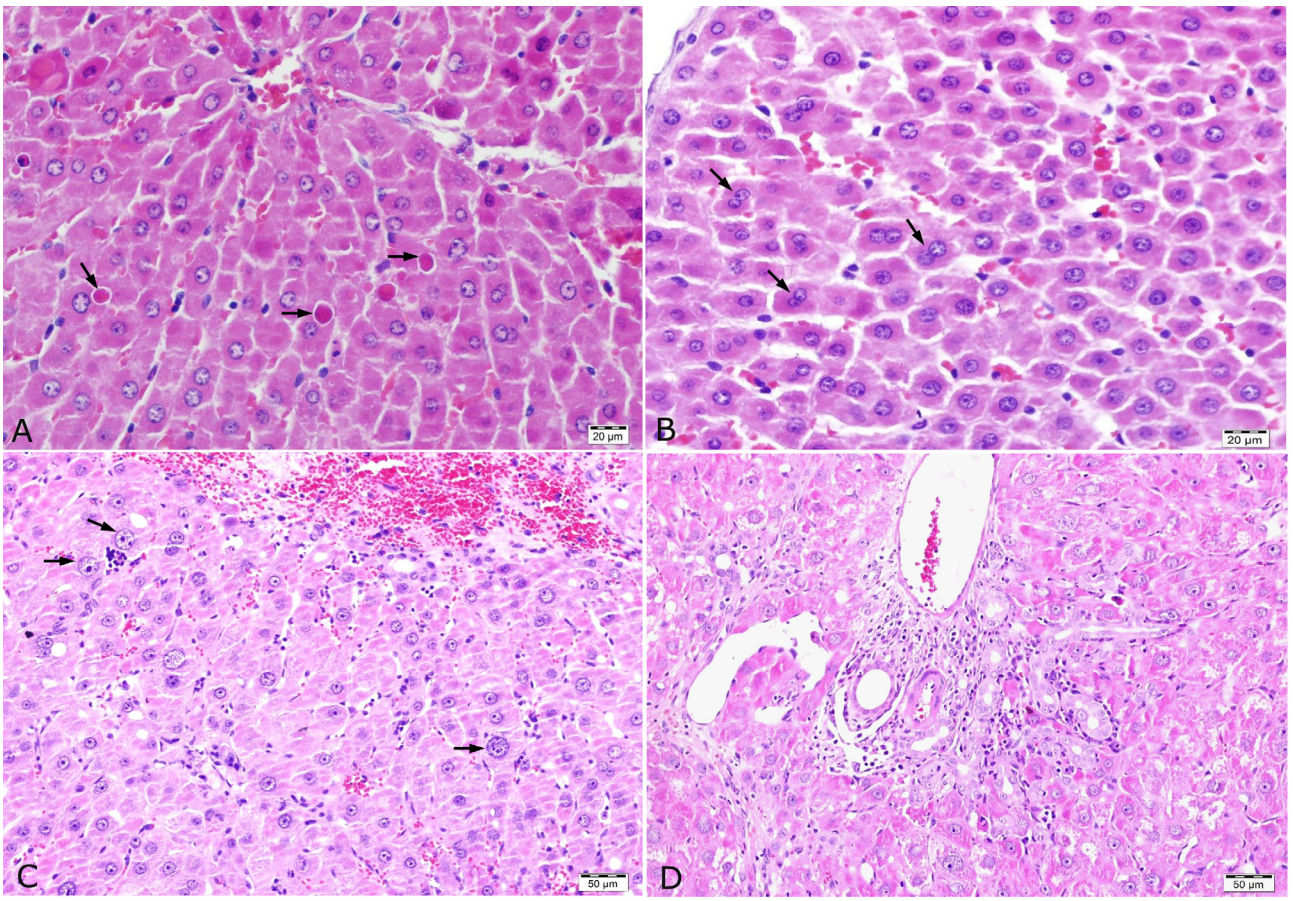

Figure 1. A- Histopathological figure shows many apoptotic cells (arrows) in the liver of a rat in Group III at the end of the 18th hour, H&E. B- Figure shows a large number of dual nucleated liver cells (arrows) of a rat in Group III at the end of the 18th hour, H&E. C- Many megalocytosis (arrows) were seen in the liver of a rat in Group IV at the end of the 6th weeks, H&E. D- Bile duct proliferatios in the liver in Group IV at the end of 6th weeks, H&E.

surrounding the necrotic areas were present in addition to fatty change in hepatocytes. The intranuclear inclusions were occasionally seen in hepatocytes in Group IV. The severity of the liver damage in this period varied depending on doses. Total liver damage in Group IV occurred 20 times more than in Group I.

3.6. Immunohistochemical findings

TUNEL positive staining was present in all dosed groups of the hepatocyte nuclei at the end of the 18th hour (Figures 2A and 2B). In parallel with the doses given in all euthanasia groups at the end of the first 18th hour, the increase in the intensity of positive staining compared to the 6th week was noted.

Table 4. Semiquantitative evaluation of histopathological findings in livers at the end of the 6th week.

	BDP	FD	PNF	CF	ICI	M	TS
Group I	0	0	0	0	0	1	1
Group II	1	1	1	1	1	3	8
Group III	3	1	3	2	2	4	15
Group IV	4	1	5	3	2	5	20
Group V	0	0	0	0	0	0	0

BDP: Bile duct proliferation, FD: Fatty degeneration, PNF: Postnecrotic fibrosis, CF: Capsular fibrosis, ICI: Inflammatory cell infiltration, M: Megalocytosis, TS: Total score.

In Bax staining, in parallel with the doses given to the experimental groups (50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg), Bax immunostaining showed that the cytoplasm of hepatocytes of the euthanatized animals were strongly positively stained at the end of the 18th hours. At the end of the 6th week, hepatocytes were slightly stained (Figures 2C and 2D).

In Bcl-2 staining, in parallel with the doses given to the experimental groups (50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg) in the Bcl-2 immunostaining, cytoplasm of liver hepatocytes were slightly positively stained at the end of the 18th hour; at the end of the 6th week, very severe positive staining was observed in the hepatocytes of the animals that were exposed to 200 mg/kg; a decrease in the severity of the staining was observed in the other groups compared to the animals in the 200 mg/kg dose group (Figures 2E and 2F).

In the PCNA immunostaining, mild staining of the hepatocytes of the animals at the 18th hour was observed, which was not different from each other in all groups. The positive staining with increasing intensity was detected depending on the given dose in the euthanasia animals at the end of the 6th weeks.

4. Discussion

Monocrotaline is a macrocyclic PA derived from the *Crotalaria spectabilis* plant [24]. In the present study, single doses of 50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg intraperitoneal monocrotaline injection were determined to cause various macroscopic, microscopic, immunohistochemical findings. Although some researchers [6] stated that monocrotaline doses of 200 mg/kg and below did not cause hepatic injury and lesions were only seen above 200 mg/kg doses. The low-dose-induced liver lesions in the present study may be due to sensitivity differences among the rat strains. Similarly, it has been reported that significant variation in PA biotransformation can exist between different rat strains [25]. Furthermore, in another study, 30 mg/kg monocrotaline was administered subcutaneously to rats and pulmonary hypertension was reported to occur after 60 days. The researchers stated that some animals in the study also caused damage to the liver and kidneys. They suggested that the changes in these organs could be caused by the disorders in the cardiovascular system generated by pulmonary hypertension [26]. Weight loss and low feed consumption may be associated with deterioration of protein and carbohydrate metabolism due to hepatotoxicity in the liver [21,27-30]. Macroscopic findings of the current study revealed excessive congestion, softening in the limbs, trimming at the edges, coconut look, visceral lobes milier and grayish color foci in the liver in parallel to the previous reports [14,28,31-34].

Megalocytosis which is defined as the growth of the cytoplasm and the nucleus has been reported as a specific finding for PA toxication in many animal species and the first finding in monocrotaline toxicity in mice [8,28,35,36]. In the present study, megalocytosis was also detected in rats of all experimental groups euthanized at the end of the 6th week. The degree of megalocytic lesions were found to be mild, moderate, or severe depending on the dose administered. Degenerative and necrotic hepatocytes were observed in Groups III and IV which was evaluated as long term period. Besides, bile duct proliferation in the liver, sinusoidal congestion, parenchymal degeneration and necrosis, mononuclear cell infiltrations were observed in the present study as were reported in previous studies [14,31,32]. Since monocrotalin causes significant sinusoidal congestion, it is also used to induce experimental sinusoidal congestion syndrome in rodents [37].

In conclusion, monocrotaline showed different effects on the liver depending on dose and duration of the exposure. Apoptotic changes were demonstrated by TUNEL and Bax stainings, which were at the highest level at the 18th hour after inoculation. The decrease in apoptotic cells at the end of the 6th week was revealed by PCNA and Bcl-2 staining. The increase in PCNA staining at the end of the 6th week can be explained by the continuation of the regeneration process in unaffected hepatocytes. Besides the decreased apoptosis

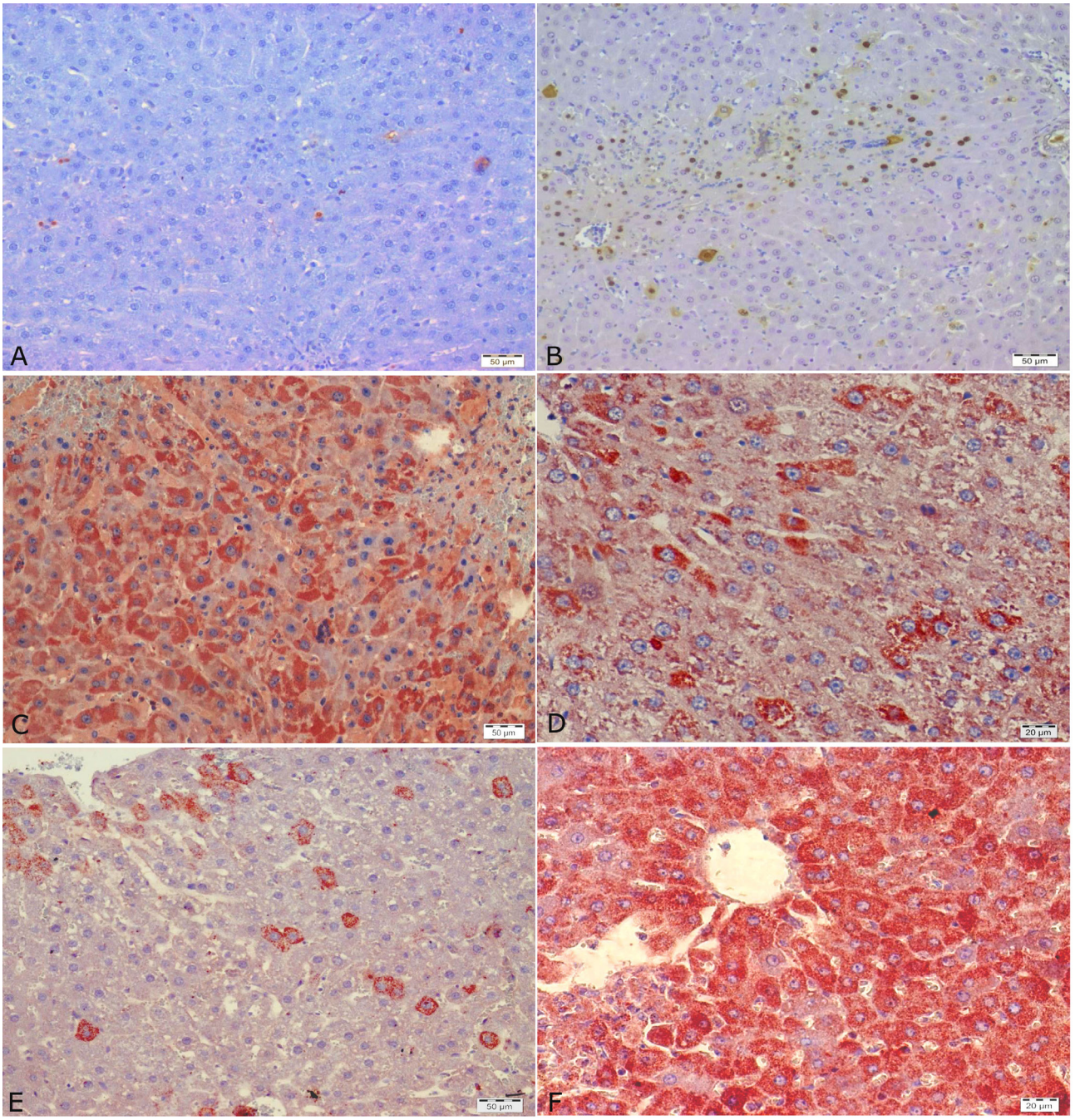

Figure 2. A- A few TUNEL positive cells were seen in the liver of a rat in Group I at the end of the 18th hour, TUNEL staining. B- Figure shows many TUNEL positive cells in the liver of a rat in Group IV at the end of the 18th hour, TUNEL staining. C- Intensive Bax positive staining was seen in the liver of a rat in Group IV at the end of the 18th hour, IHC staining. D- Moderate Bax positive staining was seen in the liver of a rat in Group IV at the end of the 6th week, IHC staining. E- Moderate Bcl-2 positive staining was seen in the liver of a rat in Group IV at the end of the 18th hour, IHC staining. F- Intensive Bcl-2 positive staining was seen in the liver of a rat in Group IV at the end of the 6th week, IHC staining.

in long-term period toxication, the mitosis was suppressed and led to the formation of megalocytosis in certain cells (increased by the given dose). Single dose of monocrotaline applied intraperitoneally to rats at doses of 50 mg/kg (Group I), 100 mg/kg (Group II), 150 mg/kg (Group III), and 200 mg/kg (Group IV) caused megalocytosis and apoptosis in liver tissue. It was concluded that the data of this study show that doses

of 100 mg/kg and above of monocrotaline significantly reduced the survival rate in 6-week trial studies. Therefore, the use of doses below 100 mg/kg may be recommended for long-term monocrotalin toxicity studies.

Acknowledgments
This study was part of Şevket SOYLU's master's thesis. The project was supported by Fırat University Scientific Research Projects (BAP) Coordinating Office (Project no: FÜBAP/ VF.14.04).

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