Impact of melamine exposure on apoptotic proteins and oxidative stress markers in mouse hepatic tissue

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ARTICLE INFO

Article History
Received : 14.09.2024
Accepted : 04.12.2024
DOI: 10.33988/auvfd.1548798

Keywords
Apoptosis
Bcl-2
Caspase-3
Melamine
Oxidative stress

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How to cite this article: Devrim AK, Devrim T, Ekici H, Sözmen M, Şenol A, Kuruca N, Sudağıdan M (2025): Impact of melamine exposure on apoptotic proteins and oxidative stress markers in mouse hepatic tissue. Ankara Univ Vet Fak Derg, 72 (2), 211-217. DOI: 10.33988/auvfd. 1548798.

ABSTRACT

Most melamine studies have focused on renal toxicity and its effects on the liver are still not well known. We investigated the apoptotic and oxidative effects of melamine on the liver using thirty BALB/c mice, divided into three groups. The control group received saline, while the low-dose melamine (LDM) group was given 400 mg/kg (1/8 LD50) and the high-dose melamine (HDM) group received 1600 mg/kg (1/2 LD50) intragastrically (0.25 ml) for 5 consecutive days. Liver Bcl-2 and caspase-3 expressions were analyzed at the protein level by immunohistochemistry and ELISA, and also at the gene level by quantitative Real-Time PCR. In addition, total antioxidant (TAS), total oxidant (TOS), and oxidative stress index (OSI) levels in liver tissues were measured spectrophotometrically. The immunohistochemical expression of caspase-3 was higher in the LDM and HDM groups compared to the control group ($p = 0.002$). TOS and OSI levels were increased significantly ($P < 0.05$) in the HDM group as compared to controls. Bcl-2 ELISA levels in the HDM group increased significantly compared to the control ($P = 0.0024$). Caspase-3 values increased significantly in the HDM group compared to the control ($P < 0.0001$) and LDM ($P = 0.0016$) groups. This study provides evidence that exposure to melamine induces oxidative stress and increases apoptosis in the liver. In conclusion, we suggest that both apoptotic and anti-apoptotic mechanisms may be disrupted at high melamine exposures, which has not been reported extensively in previous publications.

Introduction

Melamine is a nitrogen-containing organic compound, also known as 2,4,6-triamino-1,3,5-triazine. It is widely used in plastics, coatings, adhesives, and fire retardants. However, melamine can also be present as a contaminant in human and pet foods (8, 15, 20). Low melamine levels (3.0-5.12 mg/kg) in food do not cause acute symptoms, so melamine is an easily overlooked environmental toxin (6).

Melamine toxicity studies have focused mainly on renal pathological changes. So far, the toxic effects on kidney tissue have been reported in detail (11). Although the liver is the largest organ involved in the neutralization and detoxification of various harmful substances and chemicals, it is easily damaged by toxic compounds, and the reports on the effect of melamine on the liver remain restricted (18). A few studies have suggested hepatotoxic potential and reported that melamine caused notable changes in liver histopathology and the induction of oxidative stress, inflammation, and apoptosis (1, 7). Although melamine-induced oxidative stress on the total antioxidant (TAS), total oxidant (TOS), and oxidative stress index (OSI) levels analyzed in this study have not

yet been reported in the literature, Habotta et al. (19) reported that melamine-increased oxidative stress via the formation of malondialdehyde (MDA) and nitric oxide (NO) and decreased the levels of reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activities.

Caspase-3 is recognized as a crucial mediator of apoptosis, triggered within apoptotic cells through both extrinsic and intrinsic pathways. This enzyme is a pivotal zymogen in cellular apoptosis, remaining inactive until cleaved by initiator caspases during the apoptotic process (5, 28). Also, the Bcl-2 protein has important actions in organizing cell death through the regulation of apoptosis (27, 31). It is the most important anti-apoptotic protein, whose overexpression may influence chemo-resistance, DNA repair, cell proliferation, and tumorigenesis. High levels of Bcl-2 protein expression were reported in many tumors, including liver cancers (10, 32).

Exposure to toxic agents promotes the degradation of nucleic acids and disruption of apoptotic mechanisms by increasing oxidative stress products in the liver (2). Presently, our understanding of melamine's hepatic toxicity remains limited. This study aimed to delve deeper into the toxic effects of melamine on the liver in mice and evaluate the hepatotoxic, apoptotic/anti-apoptotic, and harmfully oxidative potentials of melamine on liver tissue.

Materials and Methods

Experimental design and treatment of animals: The experimental design was approved by the Ethics Committee of Animal Experiments (Meeting/Decision no. 2018-18.09/47). Thirty 10 weeks old, male BALB/c mice weighing 33.43 ± 2.63 g were equally divided into 3 groups: the negative control group given saline (control, group 1), the group receiving low dose melamine (LDM, group 2), and the group receiving high dose melamine (HDM, group 3). The specifications of the experimental environment, feeding, and care of the mice were designed as previously reported (13). Routine health checks and weighing were made after the adaptation period. Applications (0.25 ml) were made intragastrically once daily for 5 consecutive days at 10 a.m. (20). Dose calculations were based on the median lethal dose (LD50, 3200 mg/kg) of melamine (M2659, Sigma Aldrich®, USA) and the mice received 400 mg/kg (1/8 LD50) once daily in the LDM group and 1600 mg/kg (1/2 LD50) in the HDM group for 5 consecutive days (35). During the experimental period, subjects were provided with free access to food and water ad libitum. Throughout the experiment, the animals were monitored daily for body weight, behavioral changes (irritability), and signs of poisoning (hematuria).

On the 35th day after the first administration, mice were euthanized by gradually increasing concentrations of carbon dioxide inhalation (9). The livers were removed, weighed, and allocated for histopathology, biochemistry, immunohistochemistry, and Real-Time PCR analyses.

Histopathological and immunohistochemical evaluation: Liver tissues were fixed in 10 % formalin for 24 hours and embedded in paraffin wax. Subsequently, they were cut as 5 µm sections using a microtome (Leica, DE). Prepared tissue sections were stained with hematoxylin and eosin (H&E). These were examined and evaluated for apoptosis in each group. The apoptotic cells seemed to have dark eosinophilic cytoplasm and dense nuclear chromatin. They were in single-cell formation. Apparent apoptotic cell death in the liver parenchyma was determined microscopically (7).

For immunohistochemistry tissue samples embedded in paraffin wax were sectioned at 5 µm thickness and placed on Poly-L-Lysine-coated positively charged slides. They were stained on the automated immunohistochemistry staining device (Ventana Benchmark XT, Roche, Basel, Switzerland) using Bcl-2 (Rabbit polyclonal, NB100-56098, Novus Biologicals, USA, 1/100 dilution) and caspase-3 (Rabbit polyclonal, NB100-56/113, Novus Biologicals, USA, 1/200 dilution) primary antibodies. Benign tonsil tissue was used as a positive immunohistochemical control for both proteins. H&E and immunohistochemistry sections were examined under a light microscope (Nikon Eclipse Ni, Japan) by two pathologists.

The immunoreactivity of Bcl-2 was based on the stained liver sinusoidal endothelial cells (LSECs) and caspase-3 was evaluated from cytoplasmic staining of hepatocytes. Immunostaining was scored semi-quantitatively by the presence of positively stained LSECs: 0 (negative), and 1 (positive) for Bcl-2 (36). Caspase-3 immunostaining results were evaluated by modifying the method reported by Ramalho et al. (25) using the percentage of positively cytoplasmic stained cells: 0 (0-50%), +1 (51-100%). The rate of reactivity in the hepatic tissue parenchyma was evaluated at 400 final magnification using grids of 100 squares and 10 adjacent fields that together constitute an area of 0.025 mm^2.

Oxidative stress analysis: Livers were washed with saline (+4 °C) and dried on blotting paper. Afterward, they were placed in the 1 ml storage vials and stored in a deep freezer (–80 °C) until analyses were performed. Prior to analyses 140 mM KCl solution at +4 °C was added (10%, w/v) to samples and homogenized using a homogenizer (Stuart, SHM1/EURO, UK) (26). Homogenized samples were centrifuged (Nüve, NF1200R, TR) at 3000 rpm for 5 minutes at +4 °C, and supernatants were used for determining the levels of Total Antioxidant Status (TAS, mmol Trolox equiv./lt) and Total Oxidant Status (TOS,

DOI: 10.33988/auvfd.1548798

μmol H_2O_2 equiv./lt) in liver tissue homogenates. Assays were performed spectrophotometrically (MultiskanGO, Thermo, USA) using the methods described in the kits (Rel Assay Kit Diagnostics, TR). The oxidative stress index (OSI) was calculated as a percentage of the ratio of TOS levels to TAS levels and the results were expressed in the arbitrary unit (AU) (26).

ELISA analysis: ELISA tests were performed in uncentrifuged liver tissue homogenates described in detail above. Bcl-2 and caspase-3 ELISA assays were performed spectrophotometrically (Multiskan GO, Thermo, USA) using commercial kits (SunRed Biotechnology, CN) according to the manufacturer's instructions.

Quantitative Real-Time PCR: RNA was isolated from liver samples using an RNA isolation kit (74106, QIAGEN, DE). Total RNA samples were measured at 260 and 280 nm using a nanodrop spectrophotometer (MultiskanGO, Thermo, USA) and calculated by A260/A280 ratio prior to cDNA synthesis (K1671, Thermo, USA). Synthesized samples were quantified to 200 ng and used for quantitative real-time PCR (qPCR) reactions. qPCR analyses were performed using a Real-Time PCR detection system (Light-Cycler® 480 II, Roche, CH) and the suitable kit (SYBR Green I Master Kit, Roche, CH). Detected Bcl-2 and caspase-3 expressions were normalized to GAPDH as the housekeeping gene (Table 1). The obtained crossing point (Cp) values were utilized to determine the relative expressions using the equation of $2^{-\Delta\Delta Ct}$ (33).

Table 1. Oligo-primer sequences used in the qPCR analyses of the present study.

Gene	Primers (5' to 3')	Reference
Bcl-2	R: AGCCAGGAGAAATCAAACAGAGG F: CCTGTGGATGACTGAGTACCTG	*(34)* *(21)*
Caspase-3	R: GCAGTAGTCGCCTCTGAAGA F: CCTCAGAGAGACATTCATGG	*(22)* *(17)*
GAPDH	R: CTGGGATGGAAATTGTGAGG F: TGGCCTCCAAGGAGTAAGAA	*(14)* *(30)*

Statistical analysis: The data collected underwent separate statistical analyses using the Statistical Package for the Social Sciences (SPSS 20.0, IBM, USA). Significance was assessed through the Kruskal-Wallis test, followed by the examination of group differences using the Mann-Whitney U test. A significance level of $P < 0.05$ with a 95% confidence interval was employed.

Results

Clinical signs, body and organ weights: Throughout the entire experimental duration, no instances of mouse mortality were observed. Animals showed no statistically significant differences in body weight throughout the experiment. They did not exhibit behavioral changes or signs of intoxication.

Histopathological and Immunohistochemical evaluation: There were no significant differences in the liver weights among the groups. The formation of apoptotic bodies in H&E stained sections in the control, LDM, and HDM groups were evaluated. No histopathologically significant difference was observed between the liver tissues of the control, LDM, and HDM groups in terms of mononuclear cell infiltration, congestion, and focal bleeding. Scattered single-cell hepatocyte necrosis was observed in the liver tissues in the LDM (10 %) and HDM (90 %) groups. This situation was not detected in the control group (Figure 1).

No positive LSEC immunostaining was detected for Bcl-2 in the control group. There was 30 % staining in the LDM group and 60 % staining in the HDM group. We found diffuse caspase-3 staining in only one case (10 %) in the control group, and this rate was higher for the LDM (80 %) and HDM (90 %) groups.

Using the Kruskal-Wallis test, significant differences were found between the groups in terms of apoptosis ($P < 0.0001$) as well as the expression of Bcl-2 ($P = 0.016$) and caspase-3 ($P = 0.002$) immunohistochemistry. When the groups were evaluated by the Mann-Whitney U test, there was a significant increase in the presence of apoptosis in the HDM group compared to the control and LDM groups ($P < 0.0001$, $P = 0.002$ respectively). Bcl-2 expression was higher in HDM groups compared to the control groups ($P = 0.004$). Although there was no statistically significant difference ($P = 0.067$), Bcl-2 expression was higher in the LDM group compared to the control group. Caspase-3 expressions were higher in the LDM and HDM groups compared to the control groups ($p = 0.002$).

Oxidative stress analyses: TAS levels did not alter significantly between the study groups. However, TOS and OSI levels were increased significantly ($p < 0.05$) in the HDM group as compared to controls (Figure 2).

ELISA analyses: Bcl-2 levels increased significantly ($P = 0.0024$) in the HDM group compared to the controls. Caspase-3 values increased significantly in the HDM group compared to the control ($P < 0.0001$) and LDM ($P = 0.0016$) groups. The alterations of Bcl-2 and caspase-3 proteins determined by ELISA are shown in Figure 3.

qPCR analyses: mRNA expression levels of Bcl-2 and caspase-3 genes in the liver did not exhibit significant differences between the study groups (Figure 4).

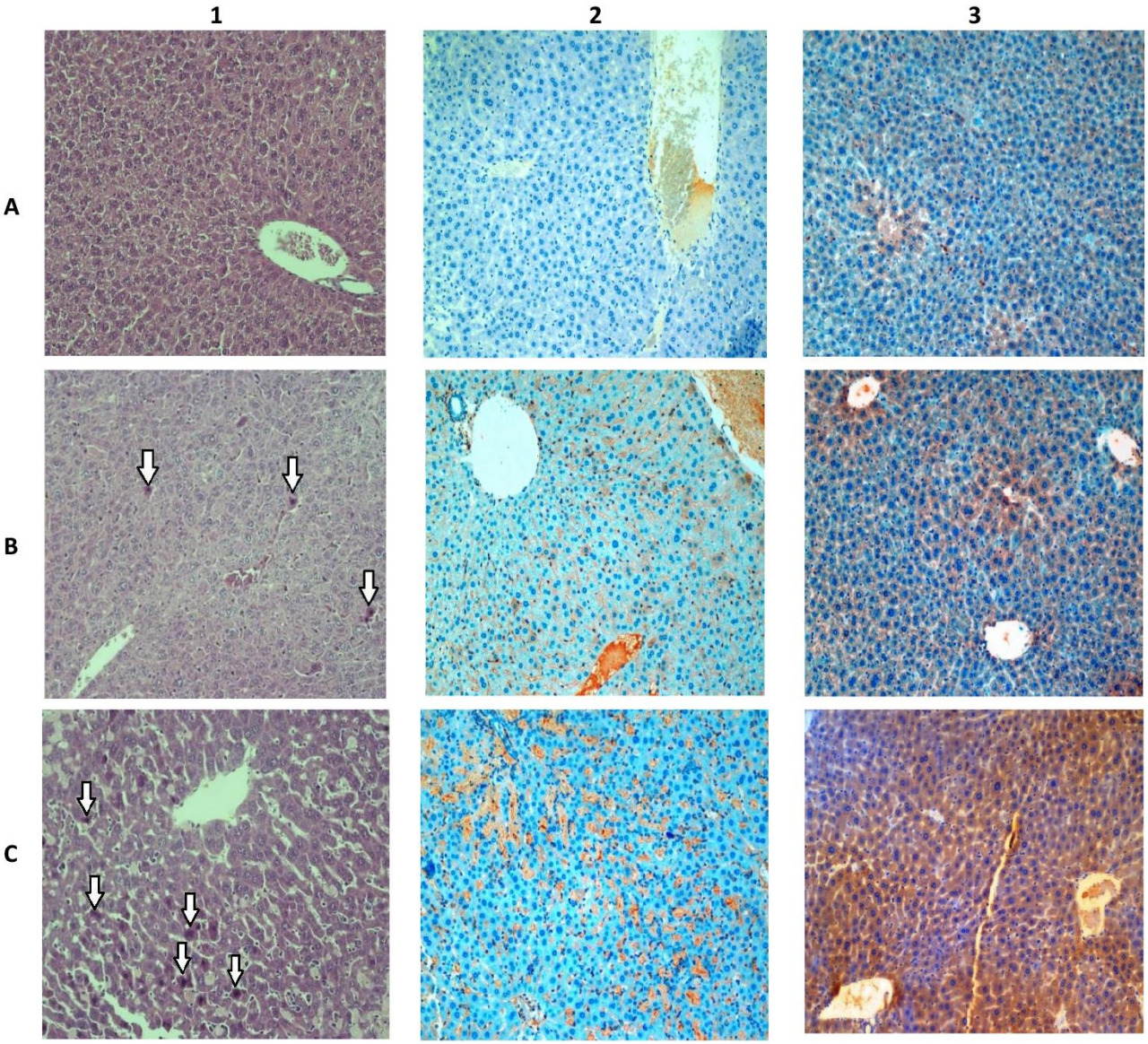

Figure 1. Microscopic images (*x200*): H&E staining (column 1), Bcl-2 immunohistochemistry (column 2), and caspase-3 immunohistochemistry (column 3) of the study groups (**A**: Control, **B**: Low dose melamine, and **C**: High dose melamine). Arrows indicate apoptotic hepatocytes.

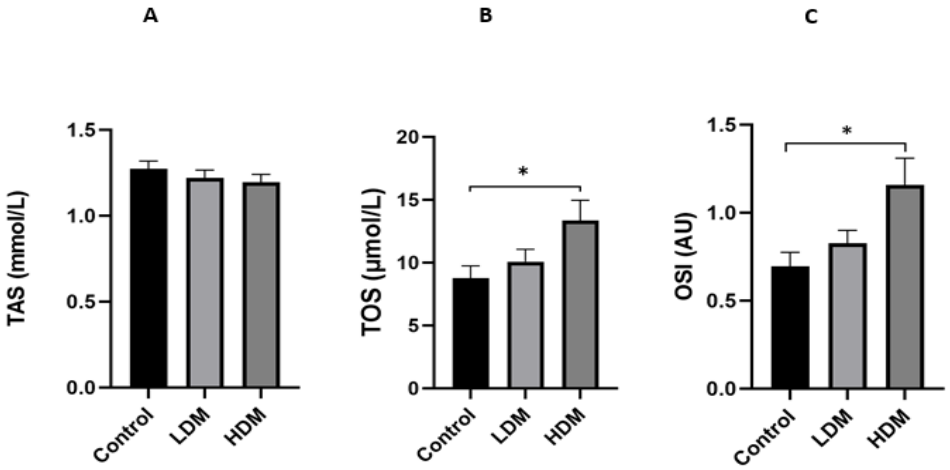

Figure 2. Levels of the oxidative stress markers (**A**: TAS, **B**: TOS, **C**: OSI) in liver tissues of control, low (LDM), and high dose (HDM) melamine applied study groups. Asterisks indicate significant (*: P <0.05) differences between the groups. Data are presented as the mean±standard error (SE).

DOI: 10.33988/auvfd.1548798

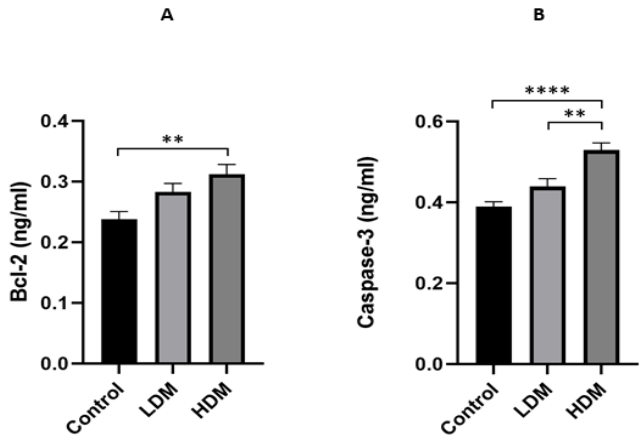

Figure 3. Results of the ELISA analyses of apoptotic markers (**A**: Bcl-2, **B**: Caspase-3) in liver tissues of control, low (LDM), and high dose melamine (HDM) applied study groups. Asterisks indicate significant (**: P <0.005, ****: P <0.0001) differences between the groups. Data are presented as the mean±standard error (SE).

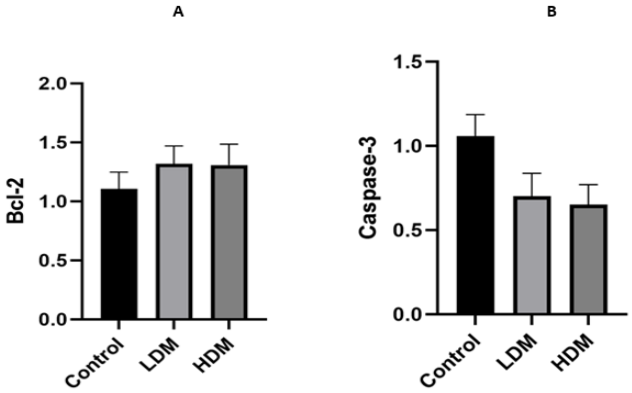

Figure 4. Quantitative Real-Time PCR (qPCR) results of the apoptotic genes (**A**: Bcl-2, **B**: Caspase-3) in liver tissues of control, low (LDM) and high dose melamine (HDM) applied study groups. Data are presented as the fold changes (mean±standard error).

Discussion and Conclusion

The histopathological examination of the present study disclosed increased levels of apoptotic cells in a dose-dependent manner in the liver tissues of all exposed groups, demonstrating that melamine could cause liver damage. Chang et al. (7) reported the pathological damage of the liver caused by orally administered 25, 50, and 100 mg/kg/day melamine. Similar to our results, they reported significantly increased levels of apoptosis even with 100 mg/kg/day melamine administration. Apoptosis, a main pathological indicator of liver injury, was also noticed in previous studies reporting cell injury induced by melamine exposure (1, 16).

As an anti-apoptotic protein, Bcl-2 supports cell survival by blocking the mitochondrial membrane pore formation (12, 27). We determined high protein values of Bcl-2 in the HDM group confirmed by ELISA and immunohistochemistry. Chang et al., (7) reported alterations in Bcl-2 levels that did not correlate with melamine doses with low (25 mg/kg) and high (50 mg/kg) melamine doses. However, they determined a Bcl-2 decrease in the melamine-treated groups. In addition, the fact that the given dose range was very low as compared to our study may be the reason for the different results obtained. Unphosphorylated Bcl-2 inhibits apoptosis, and Bax homodimers normally induce apoptosis. Bax can bind to and inhibit unphosphorylated Bcl-2, promoting apoptosis (24, 29). In our study, apoptosis in the liver increased with a rising dose of melamine, but the level of Bcl-2, which is known as the anti-apoptotic marker, also increased. Probably this was due to the binding effect of Bax, which is involved in apoptosis, to unphosphorylated Bcl-2, hence promoting apoptosis by inhibiting its anti-apoptotic effect. It is also known that post-translational mechanisms may regulate the function of Bcl-2 (23, 24). It is thought that melamine may exert an apoptotic effect by phosphorylating Bcl-2 and/or mediating Bax.

In their study investigating the protective effects of starch-stabilized selenium nanoparticles on melamine-induced hepato-renal toxicity in albino rats, Ahmed et al. (1) detected high caspase-3 protein levels at 300 mg/kg melamine-applied groups, consistent with this study. Likewise, An et al. (4) found increased caspase-3 values in melamine-applied groups as the indicator of the

progression of neuronal damage. In addition, Chang et al. (7) reported increased caspase-3 levels for the dose of 100 mg/kg/day melamine administration. A similar significant caspase-3 elevation determined by ELISA was also found in liver tissue in our study indicating liver damage.

Oxidative stress has a pivotal function in melamine-induced pathogenesis. Significantly increased levels of oxidative stress were reported by various studies inducing toxicity with oral melamine administration ≥ 100 mg/kg/day (1, 7). In the study evaluating whether Nootkatonea, naturally occurring sesquiterpenoid had a regulatory effect on oxidative stress and inflammatory liver damage triggered by 700 mg/kg melamine, it was reported that the mentioned melamine dose increased oxidative stress via the formation of MDA and NO) and decreased the levels of reduced GSH, CAT and SOD activities. In the present study, we determined high TOS and OSI values in melamine-administered groups, especially in the HDM group. Mechanisms that underlie melamine-induced liver damage are yet unclear; however, melamine exposure could evoke oxidative stress in liver parenchyma with subsequent damage and stimulating apoptosis-related proteins.

The present study assessed the pathobiochemical effects of melamine on the liver and proposed that high doses of melamine could lead to the disruption of the apoptotic mechanisms mediated by oxidative stress in liver tissue. Contrary to the reported decreasing effect of the level of 100 mg/kg, this study showed that high doses of melamine provide an increase in Bcl-2 levels, which means its anti-apoptotic effect.

In conclusion, our findings suggested that melamine toxication could cause oxidative stress-induced apoptosis in the hepatic tissue. With this study, we present evidence that both apoptotic and anti-apoptotic mechanisms may be impaired at high melamine exposures, which has not been studied extensively in previous publications, and that melamine exposure increases apoptosis in the liver by inducing oxidative stress.

Financial Support

This work was supported by the Scientific Research Projects Coordination Unit of Kırıkkale University. Project number 2019/053.

Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 2018-18.09/47).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Concept and Design: AKD, TD, HE and AS; Data Collection or Processing: AKD, TD, HE, MS, AS, NK and MS; Analysis or Interpretation: AKD, TD, HE, MS, AS, NK and MS; Literature Search: AKD, TD; Writing: AKD, TD.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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