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ORIGINAL ARTICLE

Melatonin ameliorates sodium valproate-induced hepatotoxicity in rats

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Abstract

Valproic acid (VPA) is a anticonvulsant and mood-stabilizing agent used to treat epilepsy in patients of all ages. However, it can cause hepatotoxicity with increased oxidative stress. Melatonin (MEL) is known as antioxidant and antiinflammatory agent. Therefore, the present study designed to investigate the probable protective role of melatonin against VPA-induced liver toxicity. For that purpose, 28 Wistar rats were randomly selected and divided into four groups, namely the Group C (vehicle), VPA (500 mg/kg/day VPA), MEL + VPA (10 mg/kg/day melatonin + 500 mg/kg/day VPA) and MEL (10 mg/kg/day melatonin). The agents were given by oral gavage for 14 days. Blood and liver tissue samples from all the rats were harvested on the 15th day of experiment. Biochemical analyses were conducted on the blood samples. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), alpha glutathione S-transferases (α-GST), nuclear factor-κB (NF-κB), myeloperoxidase (MPO) and changes in gene expression were examined in the liver tissues. Also, liver histopathological analyses were conducted. VPA administration significantly increased the levels of α-GST, MDA, NF-κB and of IL-1β, TNF-α gene expression in the liver compared to Group C. Moreover, vacuolization, hydropic degeneration, inflammatory cell infiltration, and sinusoidal congestion were commonly detected in the VPA-treated group along with the highest apoptotic index (TUNEL staining) values. Melatonin administration was revealed to exhibit powerful protective properties at cellular, inflammatory and oxidative level activities against VPA-induced liver toxicity. Therefore, melatonin administration may be used as an adjuvant therapy against to VPA-induced liver toxicity.

Keywords Sodium valproate · Melatonin · Hepatotoxicity

Introduction

Valproic acid (VPA) is an anticonvulsant and mood-stabilizing drug commonly used in treatment of childhood epilepsy [1]. Moreover, it is also used in adults for the therapy of epilepsy and many psychiatric disorders [2]. Despite its effectiveness and widespread use, VPA is teratogenic in both animals and humans [3]. VPA is metabolised in liver like as other xenobiotics and has widespread dose-independent hepatotoxicity on liver [4]. VPA is an eight carbon branched

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chain fatty acid and its therapeutic and toxic effects appear once it interacts with the cell membrane [5]. The mechanism of the VPA hepatotoxicity is not clear, but it is thought that fatty acids interact with mitochondrial beta-oxidation to contribute to the toxicity [6]. As long as the oxidant-antioxidant balance is maintained, organisms are not affected by free radicals. However, the loss of the balance between reactive oxygen species (ROS) and antioxidant defense mechanisms leads to critical pathological incidences resulting in physiological and biochemical dysfunctions [7]. VPA promotes the production of ROS and free radicals, which results in oxidative stress [8].

Considering the notable effects of chronic VPA administration on the quality of life and health of patients, particularly of youngs, studies are in progress for novel and effective agents have potential preventive effects against to VPA-induced hepatotoxicity [9]. Sativa oil, Vit U and sulforaphane are some agents, investigated against to hepatic damage [5, 8, 9]. However, to eliminate VPA toxicity on liver, a novel agent with no or limited side effects has been required.

Melatonin is a hormone secreted by the pineal gland [10] and has powerful free radical scavenging and antioxidant activity [11–13]. Melatonin has been also shown to reduce the toxicity of some drugs and to promote their efficacy [13]. In recent years, melatonin has been considered as a molecule having protective properties against to oxidative damage, and increasing the efficacy of the treatments [14, 15].

Therefore, the present study investigated melatonin's probable protective activities against VPA-induced liver toxicity.

Materials and methods

Animal model

Twenty-eight male Wistar rats were used in this study. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Çanakkale Onsekiz Mart University (Protocol number: 2017/04-15). During the experiment, all the rats were kept at constant temperature (22 ± 2 °C) in 12-h light/12-h dark rhythm (light, 08:00 am–08:00 pm; dark, 08:00 pm–08:00 am). The animals were fed with commercial diet and allowed to drink water ad libitum.

Experimental design

The 28 rats (300–350 g) were randomly assigned to four groups.

Group C The rats received 1 mL of 0.9% saline (n = 7).

Group VPA The rats received VPA orally (500 mg/kg/day) by gavage (n = 7).

Group MEL + VPA The rats received melatonin (10 mg/kg) 1 h prior to treatment with Valproic acid sodium salt (500 mg/kg/day) (n = 7).

Group MEL The rats received melatonin orally (10 mg/kg) (n = 7).

Melatonin (absolute GR for analysis, Cas-No-73-31-4, MERCK) was dissolved in physiological solution with 5% ethanol, final concentration included less than 1% ethanol [16] and VPA (Valproic acid sodium salt, p4543, SIGMA) was prepared in distilled water and administered daily by oral gavage according to body weight. All these treatment doses were given for 14 days. On the 15th day of the experiment, the rats were anesthetized with 5 mg/kg xylazine (Rompun®, Bayer, Istanbul, Turkey) and 50 mg/kg ketamine hydrochloride (Ketalar®, Eczacibasi, Istanbul, Turkey) with spontaneous respiration at room temperature. Blood samples were drawn by cardiac puncture under anesthesia conditions. After sacrifice, liver tissue was harvested for analysis in all groups.

Biochemical analysis

Colorimetric and enzymatic methods used to measure the levels of the blood serums ALT, AST and ALP. The liver tissues were homogenized by Qiagen Tissue Lyser II after being ground in liquid nitrogen to evaluate oxidative stress biomarkers, hepatic inflammatory cytokine and antioxidant element contents. The tissue levels of α-GST, SOD, MDA, NF-κB, and MPO were measured by ELISA. Protein concentrations were determined by the Lowry method and all the data was defined based on per mg of protein.

Genetic analysis

Total RNA was isolated from 10 to 30 mg liver tissue using a QIAamp RNA spin column (Ambion Pure Link RNA Mini-Kit) according to the manufacturer's recommended protocol. The quality and amount of the RNA were examined by determining 260/280 absorbance ratio using a NanoDrop ND-1000 Spectrofotometer.

Reverse transcription was performed using a kit (High Capacity cDNA Revere Transcription Kit). All the samples were amplified using Tagmanprobe PCRmaster mix (Applied Biosystems).

Synthesized cDNA samples were used for quantitative Real-Time PCR (ABI Stepone) study. Gene expression levels were analyzed by using Tagmanprobe. Beta-actin was used for normalization of the genes. Primer ID number of IL-1β, TNF-**α**, IL-10 and β-actin were Rn00580432_m1,

Rn01525859_g1, Rn01483988_g1, Rn00667869_m1, respectively (Thermofischer).

Histopathological evaluation

The liver tissues obtained after the completion of the surgical operation were fixed in a 10% neutral buffered formalin. The formalin-fixed tissues placed under the standard histological monitoring were embedded in paraffin blocks. Five micrometer sections were cut out of the paraffin blocks and then stained with Hematoxylin and Eosin staining. The evaluation of each section intended for vacuolization, hydropic degeneration, inflammatory cell infiltration and sinusoidal congestion, was conducted by specialized histologists with no prior knowledge about the groups (Microscope-Zeiss AxioScope.A1). The results were scored as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage.

After the evaluation, the sections from each group were imaged under Zeiss AxioScope A1 microscope equipped with Zen Blue imaging program.

Evaluation of apoptosis by TUNEL assay

After 4-µm sections were taken from the paraffin blocks to exhibit the apoptotic cells in the groups, the staining was carried out with ApopTag® Peroxidase in situ Apoptosis detection Kit (by Millipore) following the package insert of the producers. Then, 500 cells were enumerated in five areas randomly selected from the sections provided from each tissue. The cells with blue-stained nuclei were counted "normal", whereas the brown-nucleus ones were labeled "apoptotic". As a result, apoptotic index (AI) was calculated and expressed as percentage. AI = (number of TUNEL positive cells/total number of counted cells) × 100.

Statistical analysis

SPSS (Social Sciences Statistics Packages, SPSS for Windows, Version 18.0, Chicago, IC, USA) was used for data analysis. The biochemical, genetic, and histopathological results of mean and standard error values were used to present descriptive data. To analyze the differences between the groups, One-Way ANOVA followed Tukey's test were used. A p value below 0.05 was considered statistically significant.

Result

In this study, the protective activity of VPA against liver toxicity was investigated by analyzing the biochemical data and the liver gene expression levels and conducting histopathological analyses.

Biochemical data

The blood levels of ALT, AST and ALP are presented in Table 1. The significant decreases were observed in all treatment groups compared to Group C ($p = 0.05$ or $p < 0.001$) for AST levels. However, any significant change was not determined in ALT and ALP levels for all groups.

The liver tissue levels of α-GST, SOD, MDA, NF-κB and MPO are presented in Fig. 1. The lowest and significant (compared to Group VPA and MEL + VPA) α-GST level was observed in Group MEL ($p \leq 0.001$). Also, the highest α-GST level was determined in Group VPA ($p < 0.01$, compared to Group C). The liver SOD level was dramatically decreased in Group VPA ($p < 0.001$, compared to all groups). Melatonin administration partly ameliorated this decreasing in Grup MEL + VPA. The significant increasing of liver tissue MDA level was observed in Group VPA ($p \leq 0.001$). Melatonin administration significantly reduced VPA induced MDA level in Group MEL + VPA ($p \leq 0.05$). For NF-κB, the highest and only significant change was determined in Group VPA compared to MEL ($p < 0.05$). The any significant changes were not observed in MPO levels for all groups.

Gene expression levels

The results of the liver tissues expression levels of IL-1β and TNF-α were significantly increased in VPA treated group compared to Group C (Fig. 2). Melatonin administration

Table 1 Changes in the alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities in blood

Parameters	C (mean ± SE)	VPA (mean ± SE)	MEL + VPA (mean ± SE)	MEL (mean ± SE)
ALT (µ/l)	31.0 ± 1.1	35.9 ± 3.1	27.1 ± 5.4	30.1 ± 1.1
AST (µ/l)	102.1 ± 0.3	95.6 ± 1.8[a]	89.1 ± 1.4[b]	90.4 ± 2.6[b]
ALP (µ/l)	68.0 ± 1.6	79.7 ± 4.9	65.6 ± 4.3	76.1 ± 3.3

All results are presented as mean ± standard error for seven rats in each group

[a]Compared to Group C, $p = 0.05$

[b]Compared to Group C, $p < 0.001$

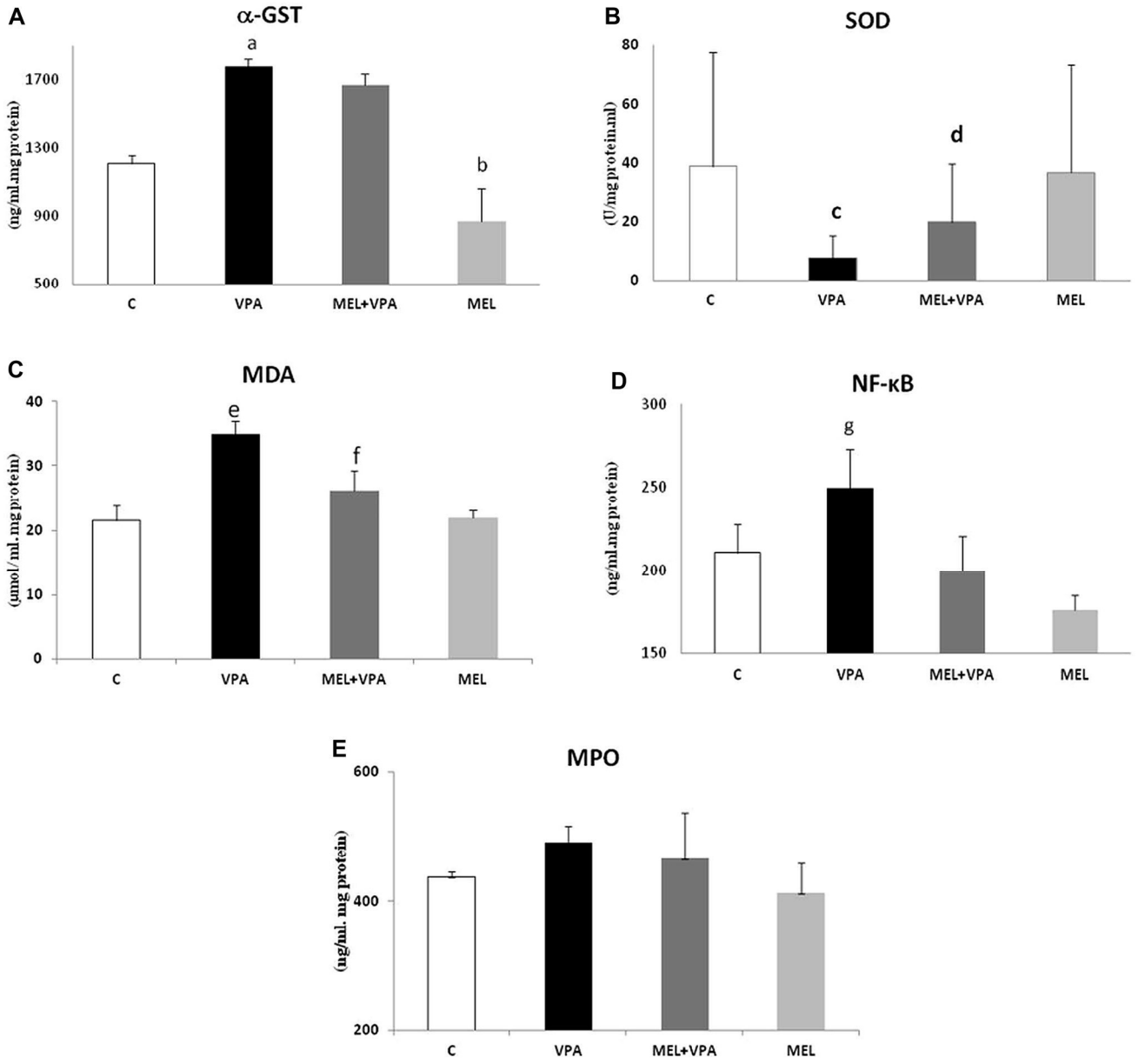


Fig. 1 The effects of VPA and MEL treatments on biochemical parameters in liver tissue between groups, enzyme activities of α-GST (**A**), SOD (**B**), MDA (**C**), NF-κB (**D**), and MPO (**E**) levels were shown, respectively. All results are presented as mean ± standard error for seven rats in each group. a: compared to Group C, $p < 0.0$; b: compared to Group VPA and MEL + VPA, $p \leq 0.001$; c: compared to all Groups, $p < 0.001$; d: compared to Group C and MEL, $p < 0.001$; e: compared to Group C and MEL, $p \leq 0.001$; f: compared to Group VPA, $p \leq 0.05$; g: compared to Group MEL, $p < 0.05$

ameliorated increasing in both TNF-α and IL-1β levels. Melatonin treatment also decreased IL-10 expression levels in both Group MEL + VPA and MEL compared to Group C ($p \leq 0.01$ and $p < 0.05$, respectively).

Histopathological evaluation

In consideration of the histopathological evaluation of the H&E sections, the light microscope evaluation of the liver tissue from Group C indicated no destruction and no other pathological modifications in the tissue architecture (Table 2, Fig. 3). In the sections from MEL + VPA, inflammatory cell infiltrations were not detected in the intralobular areas but observed to decrease substantially in the portal areas in comparison to the Group VPA. The tissue architecture was found similar to that of Group C. Vacuolization and hydropic degeneration were realized to have ameliorated in comparison with the Group VPA

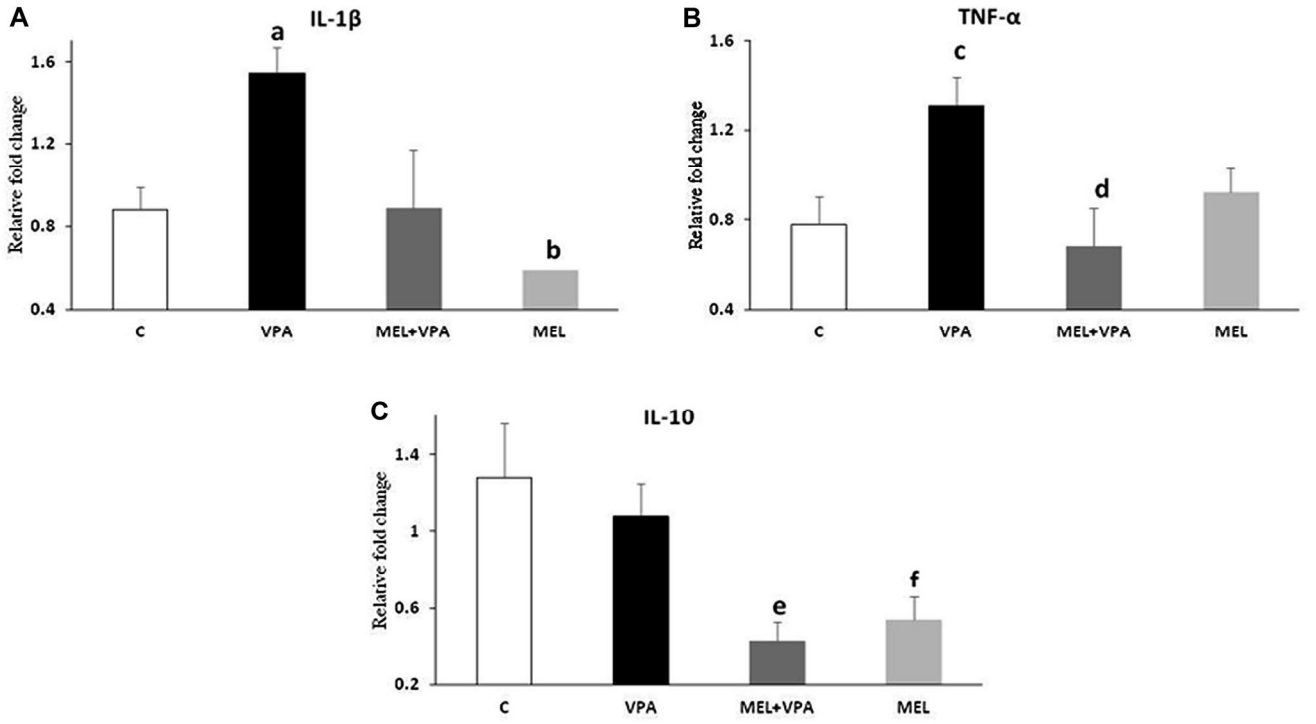


Fig. 2 Between group changes in the gene expression levels of IL-1β (**A**), TNF-α (**B**), and IL-10 (**C**). The data were presented as $2^{-(\Delta\Delta CT)}$ relative expression after the mRNA levels were normalized with β-actin. All results are presented as mean ± standard error for seven rats in each group. a: compared to Group C, $p \leq 0.05$; b: compared to Group VPA, $p < 0.01$; c: compared to Group C, $p < 0.05$; d: compared to Group VPA, $p \leq 0.00$; e: compared to Group C, $p \leq 0.01$; f: compared to Group C, $p < 0.05$

Table 2 Histopathological scores of liver tissues in all groups

Histological findings	C (mean ± SE)	VPA (mean ± SE)	MEL + VPA (mean ± SE)	MEL (mean ± SE)
Vacuolization	1.0 ± 0.0^{a}	3.0 ± 0.0^{b}	1.8 ± 0.2^{c}	1.0 ± 0.0^{a}
Hydropic degeneration	1.1 ± 0.1^{a}	3.6 ± 0.2^{b}	2.6 ± 0.3^{c}	1.0 ± 0.0^{a}
Inflammatory cell infiltration	1.0 ± 0.0^{a}	3.4 ± 0.3^{b}	2.2 ± 0.2^{c}	1.3 ± 0.2^{a}
Sinusoidal congestion	1.0 ± 0.0^{a}	3.4 ± 0.2^{b}	2.2 ± 0.2^{c}	1.1 ± 0.1^{a}

Grade result of vacuolization, hydropic degeneration, Inflammatory cell infiltration and sinusoidal congestion were scored as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage. All results are presented as mean ± standard Error for seven rats in each group

[a]Compared to Group VPA and VPA + MEL, $p < 0.01$

[b]Compared to Group C, $p \leq 0.01$

[c]Compared to all groups $p < 0.001$

and sinusoidal congestion was minimal. The image of the liver sections from the Group MEL was similar to that of Group C. The analysis of the sections from the Group VPA revealed minimal destruction in the arrangement of the hepatocyte cords and portal areas in the tissue architecture. Inflammatory cell infiltrations were observed also in the liver lobules, particularly in the portal areas in Group VPA. Sinusoidal congestion and dilatation, and vacuolization were observed to be severe. Moreover, swelling and moderate hydropic degeneration were detected in the hepatocytes, yet no focal lytic necrotic areas were observed (Fig. 3). The histopathological scoring of all groups are given in Table 2.

According to the evaluation of apoptosis by TUNEL assay, the values in the Group VPA in comparison with Group C was significantly increased to reach the highest levels among all groups ($p < 0.001$). Melatonin administration significantly decreased AI values in Group MEL + VPA compared to Group VPA ($p < 0.001$) (Fig. 4).

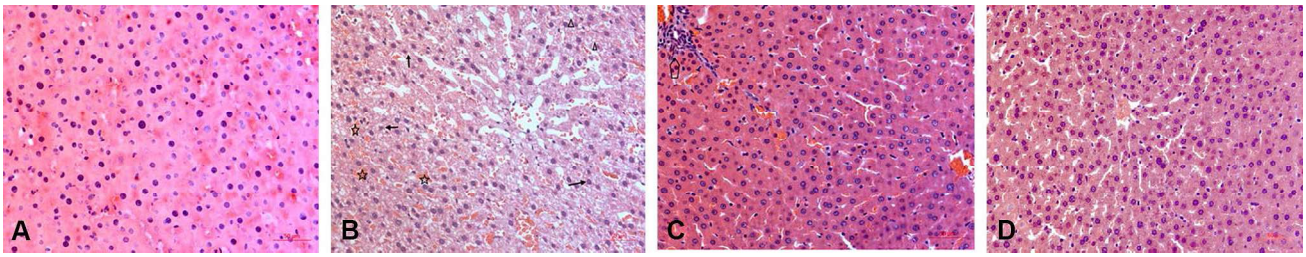


Fig. 3 Microscopic comparison of the histology of Group C, VPA, VPA + MEL, and MEL were presented in panel **A**, **B**, **C**, and **D**, respectively. (H&E staining of liver tissue, ×400 magnification). Group C showed normal histological appearance (**A**), Group VPA showed tubular necrosis and peritubular hemorrhage (arrow) and tubular epithelial desquamation and degeneration (asterisk) (**B**), Group MEL + VPA showed minimal peritubular hemorrhage (**C**), Group MEL showed similar appearance to Group C (**D**)

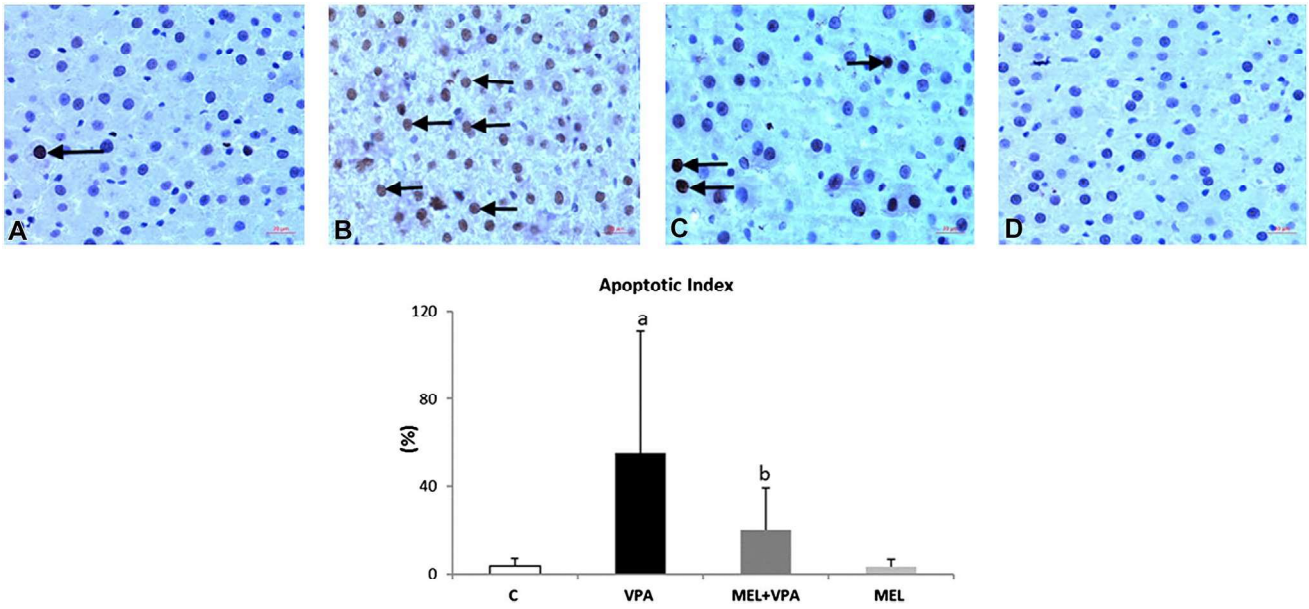


Fig. 4 TUNEL staining of of liver tissue. The by-group comparison of the apoptosis levels was provided in panel **A**, **B**, **C**, and **D**, respectively. Group C showed a few TUNEL-positive cells (**A**), Group VPA showed higher TUNEL-positive cells (**B**), Group MEL + VPA fewer TUNEL-positive cells were recorded (**C**), Group MEL (**D**), All results are presented as mean ± standard Error for seven rats in each group. a: compared to all Groups $p < 0.001$; b: compared to Group MEL and C $p < 0.001$

Discussion

The present study firstly revealed that melatonin administration has protective effects on oxidative injury, inflammation and apoptosis in VPA-induced liver toxicity.

The liver damage widely occurs following chronic VPA administration and in some cases irreversible fatal liver failure develops [5]. Therefore, preventive agents intended for liver damage should be introduced in the VPA treatment. Melatonin is a hormone secreted by the pineal gland and has powerful free radical scavenging and antioxidant activity [11–13]. Melatonin is also associated with oxidant-antioxidant balance and inflammatory processes due to its own cell-protective and anti-inflammatory properties [17]. For these reason, melatonin was investigated to reveal its own preventive feature against to VPA induced hepatotoxicity in our study.

ALT, AST, and ALP are most commonly used as serum markers to clarify liver damage known as serum aminotransferases [8]. Although these enzymes are examined as indicators of liver damage in lots of experimental and clinical studies, there are controversial results about their specificity. For example, liver necrosis may develop regardless of whether an increase occurs in the levels of these enzymes was reported [18, 19]. Thus, the levels of serum ALT and AST may not be eligible markers to evaluate the hepatotoxic effects of VPA [20]. In the present study, we also investigated these enzymes as indicators of liver damage. However, we could not obtained substantial data to evidence that VPA

causes liver damage according to ALT, ALP and AST results despite to histopathological evidence such as necrosis, inflammatory infiltration and fibrosis which indicate liver damage. Shaaban et al. observed significant increases in the levels of all the three enzymes in the rats treated with only 300 mg/kg of VPA [21]. Similarly, Nazmy et al. detected increased levels of ALT and AST after the treatment of 700 mg/kg of VPA in rats [9]. On the other hand, Lee et al. did not observe significant changes in the level of enzymes which were administered 100-500 mg/kg of VPA [18]. They proposed underlying mechanism of their result such as ALT and AST distribution to across the periportal area and having broad variability. All of these factors may be valid and acceptable in our study.

Despite the lacking of significant changes detected in ALT and ALP levels, the α-GST values significantly increased in the liver tissue after the VPA administration similarly other studies [18, 22, 23]. The melatonin administration decreased this elevation but this effect was not found statistically significant. Therefore, it can be concluded that melatonin had a protective activity on hepatic α-GST levels in VPA toxicity but its effect was remained limited. α-GST is known as a group of detoxifying enzymes and any exposure to toxic chemical increases the secretion of this enzyme [24]. Because of tending of the level of α-GST to increased in centrilobular necrosis, but ALT level did not change, α-GST was reported as a more indicative marker for centrilobular damage of liver due to their different distribution compared to ALT [25]. For that reason, we also investigated that α-GST levels in our study. However, there are limited number of studies investigating the changes in α-GST level resulting from VPA-induced liver toxicity. Lee et al. determined increasing blood α-GST levels depending of increased doses of VPA [18]. Our study differs from Lee et al. due to α-GST levels were investigated from liver tissues [18]. Moreover, many other drugs and metabolites are known to increase α-GST levels in tissues and blood as a result of the liver tissue damage [26, 27]. However there is scarcity in researches investigating the liver α-GST level after VPA administration. In this regard, our research was firstly reported elevation of α-GST levels after VPA-induced liver toxicity.

The increased levels of oxidative stress and free radicals in VPA induced hepatotoxicity were reported [21]. Human body has effective antioxidant mechanism against free radicals causing damage to liver cells. Endogenous antioxidant enzymes as α-GST, SOD and GSH and such non-enzymatic antioxidants as melatonin, transferrin, uric acid, bilirubin and albumin have antioxidant properties [7]. The protective effect of melatonin against tissue damage was reported in different studies [28–30]. Our study findings indicated that VPA-induced hepatotoxicity was associated with the increase in the lipid peroxidation and impaired the antioxidant system. However, melatonin administration strengthened the antioxidant capacity.

It is known that hepatotoxicity occurs after the emergence of inflammatory process in liver. In the event of a liver tissue damage, immune stimulation initiates NF-κB nuclear translocation and the production of inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10, IL-1ra) process [31]. IL-10 is also a powerful hepatoprotective agent in the macrophage and the T cell-mediated model of acute liver damage. Recently, it has been shown that acetaminophen caused neutrophilic infiltration, hepatocyte proliferation and liver fibrosis in rats [32]. VPA-triggered inflammatory and immune response is a process mediating the release of inflammatory cytokines initially regulated by TNF-α. Nazmy et al. found that 3.6-fold increase in the TNF-α level in the group treated with 700 mg/kg of VPA and showed that sulforaphane (0.5 mg/kg) administration recovered hepatic TNF-α level [9]. In our study, liver TNF-α and IL-1β gene expression levels were two fold increased in the VPA-administered group while these values in MEL + VPA administered group were found as close to Group C. These results indicated us melatonin has decreasing effect on inflammatory process induced by VPA in liver tissue. Marcondes-Alves et al. administrated kaurenoic acid extract for inhibition of acetaminophen-induced hepatotoxicity by reducing oxidative stress and the production of proinflammatory cytokines. Their results suggested that kaurenoic acid may be used as preclinical agent in acetaminophen-induced hepatotoxicity and lethality [33]. Likewise, our results indicated that melatonin may defined as a preclinical agent against to VPA-induced liver toxicity. Melatonin also decreased NF-κB level in liver tissue which is another cytokine has role in liver toxicity [34]. This result strengths our suggestion related with melatonin usage in preclinical level in liver toxication.

In our study, inflammatory cell infiltrations, vacuolization, hydropic degeneration, sinusoidal congestion and increased apoptotic cell number of liver were observed in VPA-administered group. Nazmy et al. observed similar histopathological findings also focal necrotic area in VPA administrated rats [9]. They administered sulforaphane after the VPA toxicity and found reduced hepatotoxicity, decreased neocholangioles, portal inflammatory areas and focal lytic necrosis [9]. Our histopathologic findings are similar to Nazmy et al. except for focal necrosis. The absence of focal necrosis following to VPA administration may be due to the duration of VPA treatment and tissue harvesting period, conversely Nazmy et al. study, we applied short term VPA toxication. However, melatonin significantly decreased liver damage in VPA-induced toxicity similarly to sulforaphane. These results indicated us melatonin is an agent has powerful effect against to

VPA-induced liver toxicity in histopathological level and may has potential preclinical usage.

Apoptotic index is another important parameter in evaluation of VPA-induced liver toxicity which stands for measure of the extent of apoptosis. We observed higher AI result in Group VPA. However, melatonin administration has a protective role against to increased AI level induced by VPA. El Mowafy et al. investigated that protective role of Docosahexaenoic acid (DHA) in the case of a VPA-induced liver toxicity [35]. They observed a higher number of TUNEL positive apoptotic cells in the VPA and DHA treatment significantly decreased apoptotic cells number in VPA-DHA group. Similarly, the present study found a significantly higher number of apoptotic cells determined with the AI values in the Group VPA than in the Group C. Melatonin was observed to have reduced the AI values in the Group MEL + VPA and to have led to a promoted cellular viability.

Conclusion

These outcomes indicate that melatonin has antioxidant, anti-inflammatory and anti-apoptotic properties in VPA induced liver toxicity. Melatonin is a highly reliable molecule with almost no side effects. Therefore, it can be used as a protective agent against hepatotoxicity caused by VPA.

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Compliance with ethical standards

Conflict of interest All authors declares that they have no conflict of interest.

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