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ORIGINAL ARTICLE

Hepatoprotective actions of melatonin by mainly modulating oxidative status and apoptosis rate in lipopolysaccharide-induced liver damage

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ABSTRACT

Aim: One of the serious complications of sepsis is liver damage and liver failure. This study aimed to evaluate the protective and therapeutic potential of melatonin in rats with lipopolysaccharide-induced sepsis.

Main methods: Female Spraque–Dawley rats received single a dose of 7.5 mg/kg lipopolysaccharide in saline to create a 24-h sepsis model. One of the other groups received melatonin at a dose of 10 mg/kg/day beginning 1 week before sepsis induction to the end of the experiment. The melatonin group received the same doses of melatonin for the same duration but not lipopolysaccharide. The vehicle group received the same doses of saline, the vehicle of melatonin, for the same duration. Twenty-four hours after the last injection, the rats were decapitated. By appropriate histochemical, immunohistochemical, biochemical, and molecular techniques, anti-necrotic, anti-apoptotic, anti-necroptotic, anti-inflammatory, and antioxidant effects of melatonin were assessed.

Key findings: Lipopolysaccharide has disrupted liver functions by inducing oxidative stress, inflammation, necrotic, apoptotic, and necroptotic cell death, thus disrupting liver functions. Melatonin was found to be beneficial in terms of inhibiting the intrinsic pathway of apoptosis and tissue oxidant levels, stimulating tissue antioxidant enzyme levels, and restoring hepatocyte functions.

Significance: Melatonin, at those doses and duration, was found to be hepatoprotective by mainly modulating oxidative status and apoptosis rate, however, failed to significantly reduce histopathological damage. We suggest that longer-term melatonin administration may produce anti-inflammatory and anti-necrotic effects as well.

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KEYWORDS
Apoptosis; necrosis; necroptosis; liver; melatonin; oxidative stress

Introduction

In the syndrome of sepsis, an infection-related dysregulation of the host's immune system results in numerous life-threatening organ failure [1]. The syndrome has a significant death rate due to its complex pathogenesis and few therapy choices. Sepsis is caused mainly by gram-negative bacteria containing lipopolysaccharides (LPS) on their outer membrane [2]. LPS interacts with various plasma proteins and activates various immune-competent cells. Tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-8, and nitric oxide are only a few of the pro-inflammatory mediators released by activated cells that possess a crucial role in the pathophysiology of sepsis [3]. Therefore, researchers have adopted a strategy to develop new protocols for the treatment of LPS-initiated organ damage in terms of therapeutic removal or neutralization of LPS and/or elimination of molecules mediating the activity of LPS [4].

A pineal hormone known as melatonin, which is also synthesized in extra-pineal tissues including the heart, liver, placenta, skin, kidney, and gut, is produced, and released by the circadian rhythm [5]. According to previous studies, melatonin possesses anti-inflammatory and antioxidant properties. Melatonin is thought to function primarily by scavenging free radicals, activating natural antioxidant enzymes, and enhancing the effectiveness of other antioxidant substances [6,7]. Additionally, by modifying the mitochondrial membrane potential, melatonin shields the mitochondria from oxidative damage [8]. Growing data support melatonin's anti-inflammatory effects in both short-term and long-term inflammatory disorders. IL-1, TNF, and other pro-inflammatory cytokines were found to be decreased after melatonin administration in animal tests, whereas serum levels of the anti-inflammatory cytokine, IL-4, were increased [9,10].

One of the key organs in charge of the body's detoxification processes is the liver. The three major molecular processes that LPS-induced endotoxemia generates are hepatic

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inflammation, apoptosis, and oxidative stress [11,12]. Various cell death pathways, including necrosis, apoptosis, necroptosis, NETosis, pyroptosis, and autophagy-induced cell death, can be activated during sepsis [12–14]. So, using appropriate histochemical, immunohistochemical, western blotting, and biochemical techniques, we sought to study the antioxidant, anti-necrotic, anti-apoptotic, anti-necroptotic, and anti-inflammatory effects of melatonin on LPS-induced liver damage.

Materials and methods

In the present study, 42 female Spraque–Dawley rats, aged 4–6 months, weighing 150–250 grams were used. The rats were kept in a room-temperature storage facility at 25 °C with a 12:12 light/dark cycle, *ad libitum* access to water, and a regular meal. Just before the first injection and decapitation, the body weights of the rats were recorded.

Experimental design

Rats randomly were divided into four groups as follows: the vehicle group (V group) ($n = 8$), LPS-administered group (LPS group; $n = 13$), LPS and melatonin-administered group (LPS + Mlt group; $n = 13$), and melatonin-administered group (Mlt group $n = 8$). LPS in saline was injected intraperitoneally as a single dose of 7.5 mg/kg to the rats of the LPS groups for establishing a 24-h sepsis model [15,16] (*Escherichia coli*-serotype O111:B4; Sigma-Aldrich, St. Louis, MO, USA). LPS + Mlt group received melatonin (M5250; Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally at a dose of 10 mg/kg/day [17] beginning 1 week before LPS administration to the end of the experiment. Melatonin was dissolved in ethanol and diluted in saline until 1% concentration. The Mlt group received the same dose of melatonin for the same duration but did not receive LPS. The vehicle group received the same doses of saline for the same duration. Twenty-four hours after the last injection, the rats were decapitated under 50 mg/kg ketamine hydrochloride and 10 mg/kg 2% xylazine hydrochloride anesthesia. Cardiovascular puncture was used to collect blood samples for biochemical examination immediately before sacrifice. The abdominal cavities of the rats were exposed, and their livers were excised immediately. Half of the tissues were placed in 10% neutral buffered formaldehyde for the histopathological examination, remaining tissues were preserved at −80 °C for biochemical and western blotting investigation.

Histopathological evaluation

After fixation, tissues were dehydrated using a succession of alcohol solutions with progressively higher alcohol content, cleaned by passing through xylol and then embedded in paraffin. Hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS)-stained 5-m slices were used for microscopic analysis. All samples were examined with a Nikon Eclipse i5 (Tokyo, Japan) with Nicon DS-Fi1c camera attachment (Tokyo, Japan) and analyzed by NIS Elements version 4.0 image analysis system (Nikon Instruments Inc., Tokyo, Japan). Histopathological changes were evaluated and graded. Necrosis and inflammation (they occurred together, and the severity of both correlated) were graded together as 0: 1%–5% of 10 areas; 1: 26%–50% of 10 areas; 2: 51%–75% of 10 areas; 3: 76%–100% of 10 areas under 10× magnification. Sinusoidal dilatation and vacuolization were graded separately as 0 = none; 1 = mild; 2 = medium; 3 = severe. The maximum score was 9.

Immunohistochemistry

Ki-67 (Merck, USA), caspase-8 (Abcam, UK), and OV6 (R&D Systems, USA) detection kits were used for detecting proliferation, apoptosis, and stem/progenitor cell status, respectively. Staining techniques were performed according to the instructions of the manufacturers. Fifteen successive areas in each section were examined for the presence of positive cells at 20× magnification under the light microscope. All the analysis and imaging procedures were performed using Nikon Eclipse i5 microscope and Nikon NIS Elements version 4.0 imaging and analysis system (Nicon Instruments Inc., Tokyo, Japan).

Western blotting analysis

Almost 100 mg of liver tissue from each animal was added to Ristosetin agregation test (RIPA) lysis buffer and homogenized with metallic beads after being rinsed with 0.1 M phosphate-buffered saline (PBS). Then, homogenized tissues were centrifuged at 14,000 rpm for 15 min at 4 °C. Electrophoresis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the collected supernatants. Equal amounts of protein (40 µg/µl) which were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham) by Multiskan™ GO Microplate Spectrophotometer were diluted in Laemmle sample buffer (Bio-Rad Laboratories, Inc., USA), boiled for denaturation of proteins and loaded onto 4%–20% Bis-Tris polyacrylamide gels and transferred into polyvinylidene difluoride (PVDF) membranes using Bio-Rad protein electrophoresis and blotting system. Membranes were incubated in blocking solution (5% milk powder in 0.1% Tween 20/0.1 M Tris-buffered saline (TBST) and immersed with primary antibodies including Bcl-2 (Cell Signaling, Danvers, USA), Bax (Cell Signaling, Danvers, USA), RIP-3 (Abcam, Boston, USA), mixed lineage kinase domain-like pseudokinase (MLKL) (Abcam, Boston, USA), IL-1β (Novus Biologicals, USA), and NF-κB (Cell Signaling, Danvers, USA). Each antibody was diluted 1:1000 in 5% milk powder, 0.1% Tween 20, and 0.1 M TBST before being incubated overnight with the membrane while being shaken. The membranes were washed the next day and then incubated with secondary antibodies that had been peroxidase-coupled and diluted in the same solvent as the primary antibodies. Signal detection was made with luminol substrate (Advansta, San Francisco, USA) under a CCD camera with Fusion FX7 system (Vilber Lourmat). Protein loading was controlled with a monoclonal mouse antibody against GAPDH (Cell Signaling, Danvers, USA). Using the Image J analysis

system, immunoreactive protein bands were quantified densitometrically (NIH, Washington, DC, USA).

Biochemical evaluation

All tissue samples were homogenized in RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 50 mmol/l NaF, 0.1 mmol/l Na_3VO_4), and proteinase inhibitor cocktail (Merck KGaA, Darmstadt, Germany) by using bead homogenization system (BeadBug3 Benchmark Scientific NJ, USA). The final supernatant was used as the total protein after centrifugation at 14,000 rpm (Beckman Coulter, Krefeld, Germany) for 10 min at 4 °C. The Bradford method [18] was used to determine protein concentrations.

Serum sample preparation

Blood samples were drawn into the proper tubes, and centrifuged at 3000× *g* for 15 min, and the supernatants of each sample were then frozen at −80 °C until the experiments began.

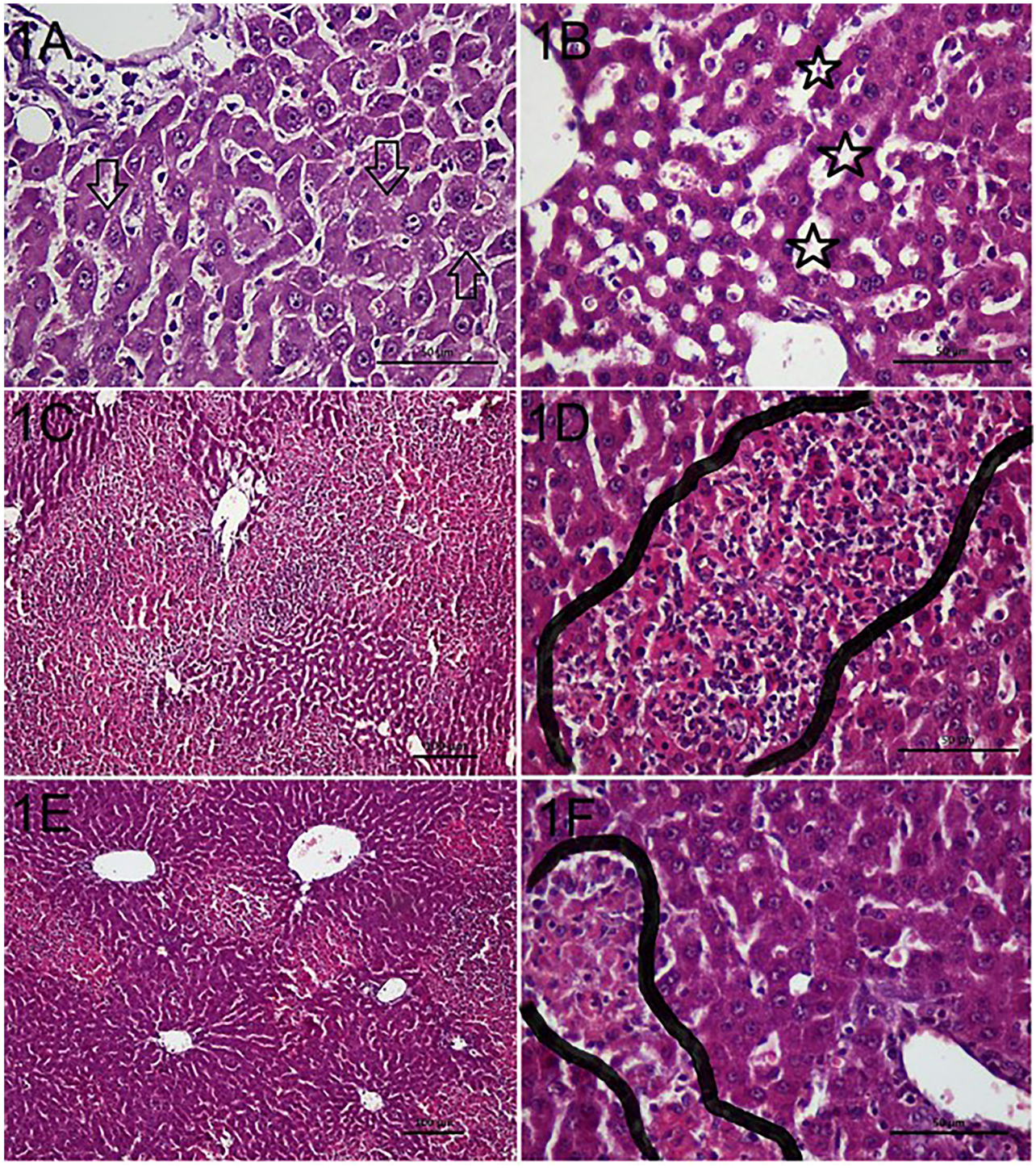

Figure 1. (A–D) Vacuolization (arrows), sinusoidal dilatation (asterisks), inflammation, and necrosis (lined by the black line) are observed in the sections from the LPS group. (E, F) Reduced inflammation and necrosis are detected at the sections from the LPS + Mel group. H&E technique.

Oxidative stress markers

All samples were stored at −80 °C until experiments started. Malondialdehyde (MDA), glutathione, superoxide dismutase (SOD), and catalase (CAT) levels in serum and tissue samples were measured by commercially available kits. E1759Ra (BT Laboratory, Hangzhou, China) was used for glutathione, E0168Ra (BT Laboratory, Hangzhou, China) was used for SOD, E0156Ra (BT Laboratory, Hangzhou, China) was used for MDA, E0869Ra (BT Laboratory, Hangzhou, China) was used for Catalase, 105134 (Abcam UK). Total antioxidant status (TAS) and Total oxidant status (TOS) levels were measured using commercially available kits (Relassay, Turkey) according to Erel's assay [19,20]. Oxidative status index (OSI) was defined as TOS to TAS ratio was calculated as follows:

$$\mathrm{OSI}(\text{arbitrary unit}) = \left[\frac{\mathrm{TOS}, \mu\mathrm{mol}\,\mathrm{H_2O_2}\,\frac{\text{equivalent}}{\text{mg}}\,\text{protein}}{(\mathrm{TAS}, \mu\mathrm{mol}\,\mathrm{Trolox}\,\frac{\text{equivalent}}{\text{mg}}\,\text{protein})} \times 100 \right]$$

ALT- and AST-level assays

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels were measured by commercially available kits (Abcam-105134, UK; Abcam-105135, UK, respectively). According to the manufacturer's instructions, an analysis was conducted.

Statistical analysis

Descriptive statistics of the data: the mean ± standard deviation was explained as medium (minimum–maximum). The Shapiro–Wilk test was used to examine whether the data had a normal distribution. The Kruskal–Wallis test was employed to compare the group's means. Dunn's test was employed as a *post hoc* test for multiple comparisons. Statistical analyses of data were done with SPSS 28.0 (IBM, New York, USA). Data with $p < .05$ were considered statistically significant.

Results

Histopathological analysis

Sections stained with H&E revealed significant histopathological alterations including vacuolization, sinusoidal dilatation, inflammation, and necrosis in the LPS group (Figure 1(A–D)). Melatonin reduced these alterations without significant impact (Figure 1(E,F)). The mean histopathological scores were 5.09 ± 2.2 in the LPS group, 3.90 ± 1.6 in the LPS + Mlt group, 1.37 ± 1.3 in the V group, and 1.57 ± 0.9 in

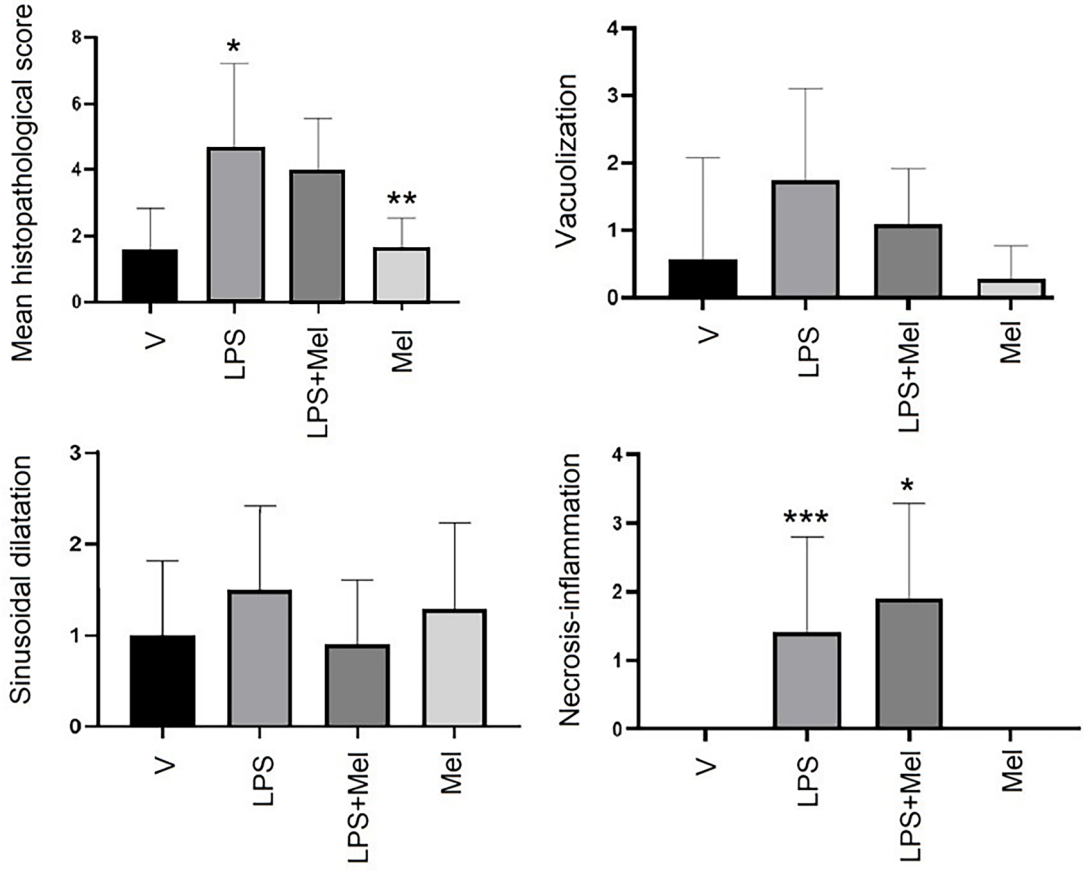

Figure 2. Mean histopathological scores and degree of vacuolization, sinusoidal dilatation, inflammation, and necrosis of all groups are summarized. *$p < .005$ versus V, **$p < .005$ versus LPS + Mel, ***$p < .05$ versus V.

the Mlt group. Melatonin reduced the main histopathological score without significant importance. Melatonin affected the severity of vacuolization and sinusoidal dilatation but not inflammation and necrosis (Figure 2).

Immunohistochemical analysis

As the results of stereological analysis, it was detected that LPS significantly reduced the proliferation rate (139.8 ± 14.8 vs. 248.8 ± 20.3; $p < .005$) (Figure 3(A–C)), melatonin administration has not completely restored the proliferation rate (139.8 ± 20.3 vs. 154.3 ± 40.4) (Figure 3(A,D)). Additionally, the mean Ki-67 positive cell number of the melatonin melatonin-administered group was lower than that of the control ($p < .005$) (Figure 1(A,E)). LPS slightly induced caspase-8 positive cell numbers (123.2 ± 65.3 vs .132.3 ± 22.7) (Figure 4(A–C)). Melatonin has not reduced the mean caspase-8+ cell numbers (Figure 4(A,D)). Melatonin alone has not affected the apoptosis rate (Figure 4(A,E)). Many OV6+ cells were detected at the epithelium of the bile ducts including canals of Herring in the samples obtained from all groups. Some hepatocytes at the border of the portal area also showed a positive

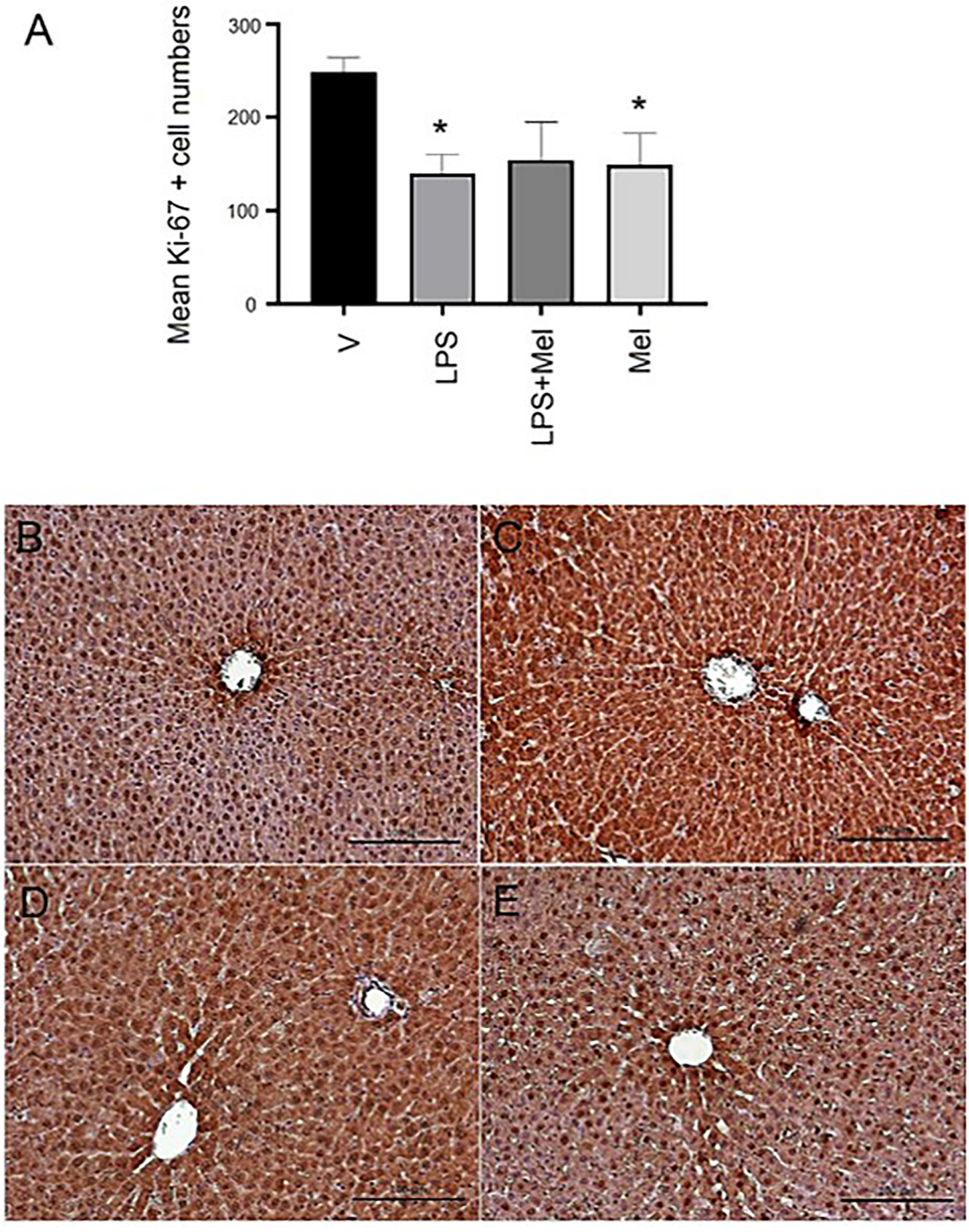

Figure 3. (A) The mean Ki-67+ cell numbers of all groups are summarized. Pictures represent the degree of immunohistochemical staining positivity of vehicle (B), LPS (C), LPS + Mel (D), and Mel (E) groups. The most powerful staining is detected in the V group, whereas positive staining seems to be lower than the other groups in the LPS group. $p < .005$ versus V.

reaction. Any differences in terms of distribution and staining intensity were detected (Figure 5(A–D)).

Western blotting analysis

The tissue IL-1 β level of the LPS group was higher than that of the vehicle group ($p < .001$). Melatonin administration has not significantly lowered that value. Tissue IL-1 β level was lower than those of the vehicle and LPS group (($p < .001$) (Figure 6(A)). Tissue NF-κB of the LPS group was also higher than that of the vehicle group ($p < .001$). Melatonin has not affected that value. Melatonin alone induced tissue NF-κB levels (Figure 6(B)). The Tissue Bax/Bcl level of the LPS group was higher than that of the vehicle ($p < .001$). Melatonin administration lowered that value ($p < .005$). The tissue RIP-3 value of the LPS group was higher than that of the vehicle ($p < .005$). Melatonin administration did not normalize that value. The tissue MLKL level of the LPS group was higher than that of the vehicle ($p < .001$). Melatonin administration reduced but did not significantly affect that value. Melatonin

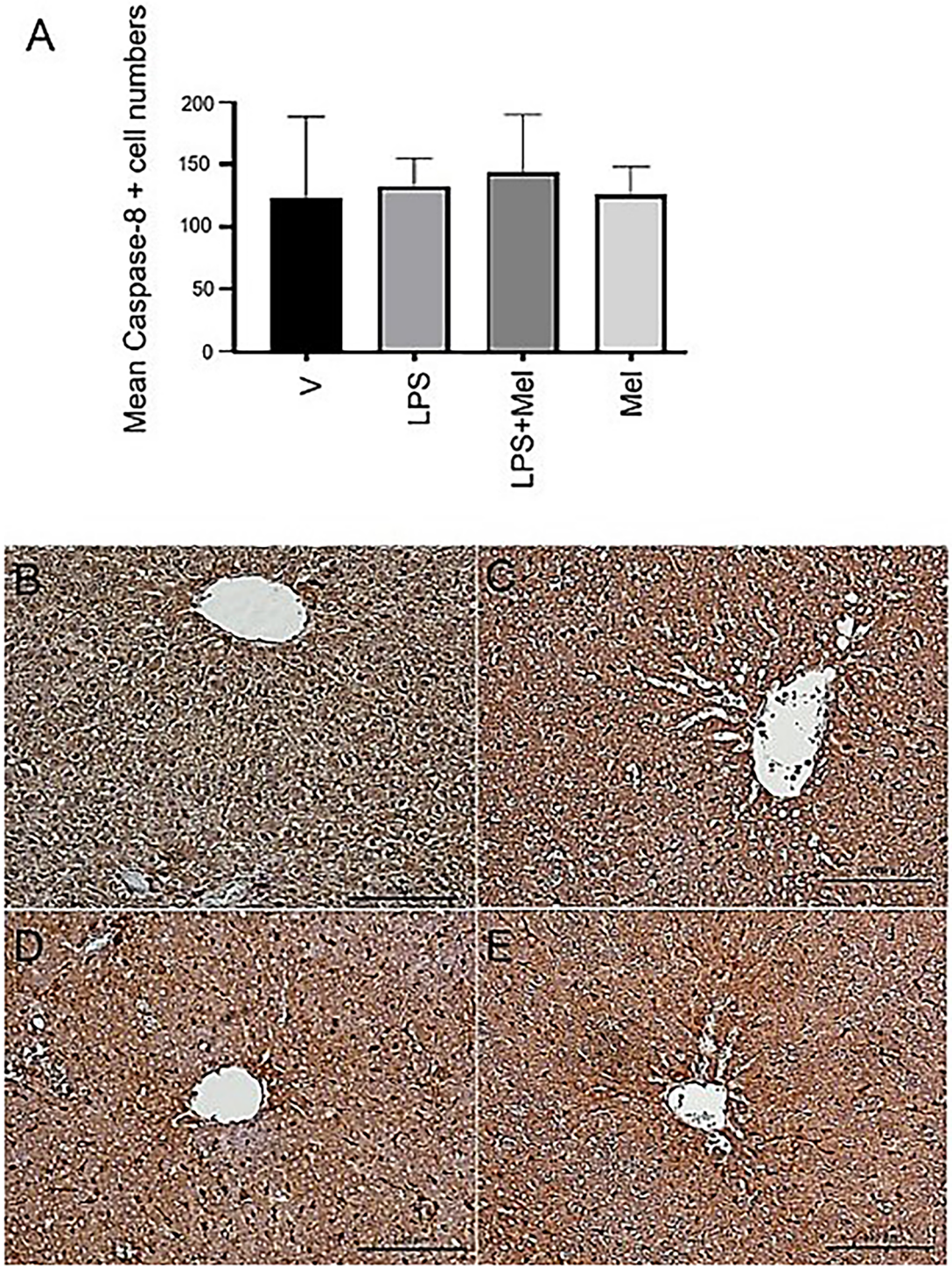

Figure 4. (A) The mean caspase-8+ cell numbers of all groups are summarized. Pictures represent the degree of immunohistochemical staining positivity of vehicle (B), LPS (C), LPS + Mel (D), and Mel (E) groups. The staining pattern is similar among groups.

alone seemed to induce tissue MLKL level versus that of the vehicle group ($p < .005$) (Figure 7).

Tissue oxidant/antioxidant analysis

The mean tissue MDA level of the LPS group was significantly increased versus the control group ($p < .001$). Melatonin slightly reduced that value. The mean serum TOS value of the LPS group was higher than that of the control group ($p < .001$). Melatonin reduced that value ($p < .001$). The mean serum TAS value of the LPS group was lower than that of the control group ($p < .001$). Melatonin significantly restored that value ($p < .001$). The mean TOS/TAS oxidative stress index of the LPS group was higher than that of the control group (5.23 ± 1.04 vs. 15.72 ± 2.14; $p < .001$). Melatonin significantly reduced that value ($p < .001$). Mean serum values of TOS, TAS, and TOS/TAS oxidative stress index of only melatonin-administered group were similar to those of the control group (Figure 8).

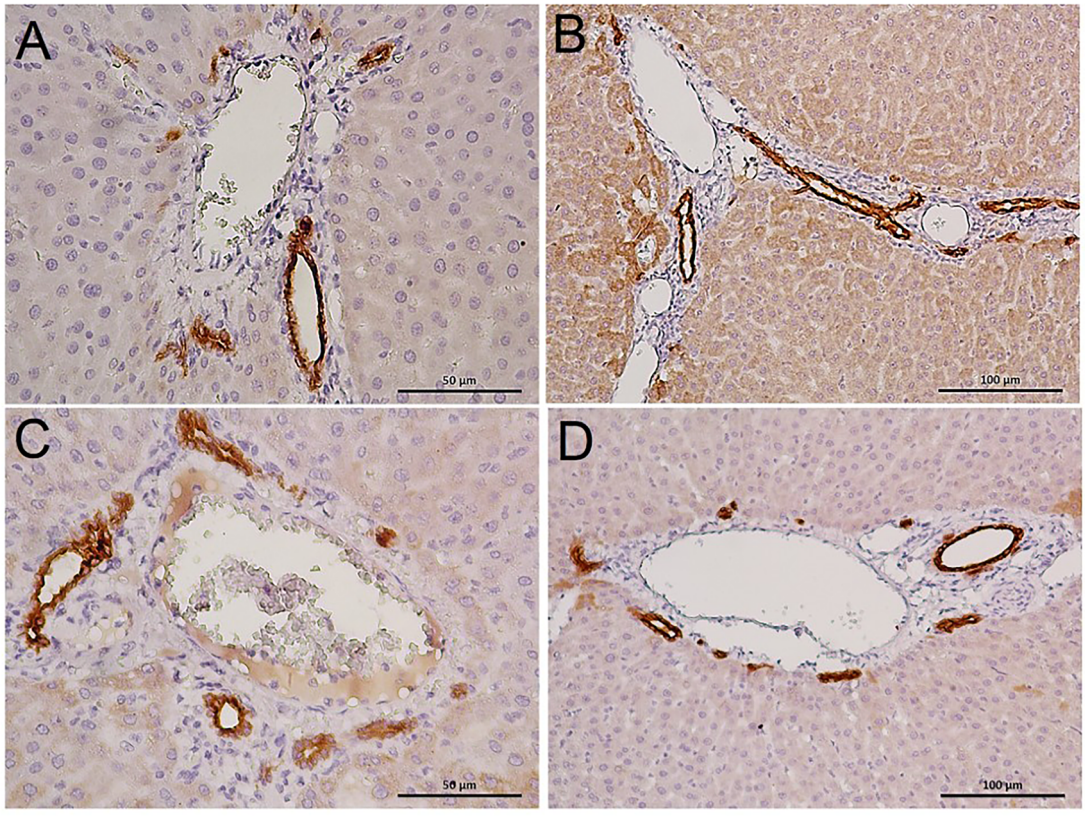

Figure 5. Pictures represent the degree of immunohistochemical staining positivity in terms of OV6 in the vehicle (A), LPS (B), LPS + Mel (C), and Mel (D) groups. OV6+ cells are located at the epithelium of the canals of Herring and bile ducts in the portal area. The staining pattern is similar among groups.

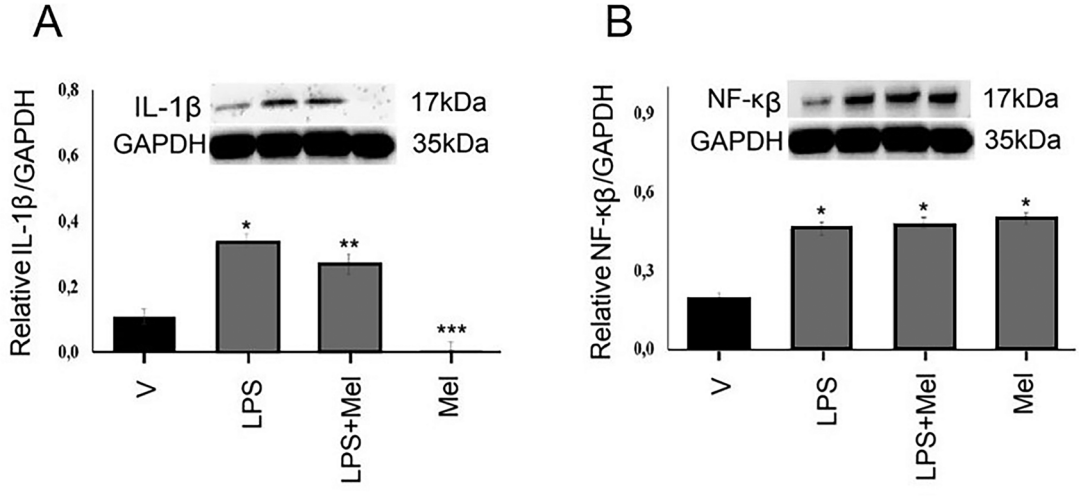

Figure 6. Results of western blotting technique in terms of tissue IL-1β and NF-κB levels. *$p < .001$ versus V, **$p < .005$ versus V, ***$p < .001$ versus LPS.

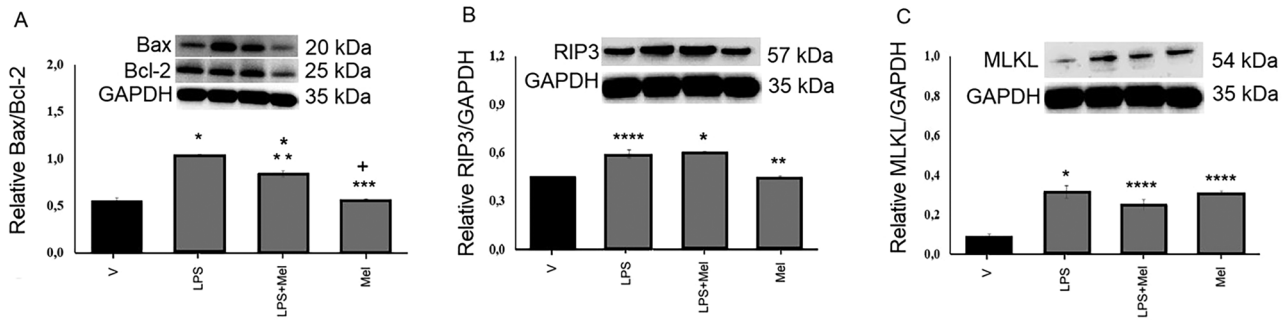

Figure 7. Results of western blotting technique in terms of tissue Bax/Bcl-2, RIP-3, and MLKL levels. *$p < .001$ versus V, **$p < .005$ versus LPS, ***$p < .001$ versus LPS, ****$p < .005$ versus V, $^{+}$<.001 versus LPS + Mel.

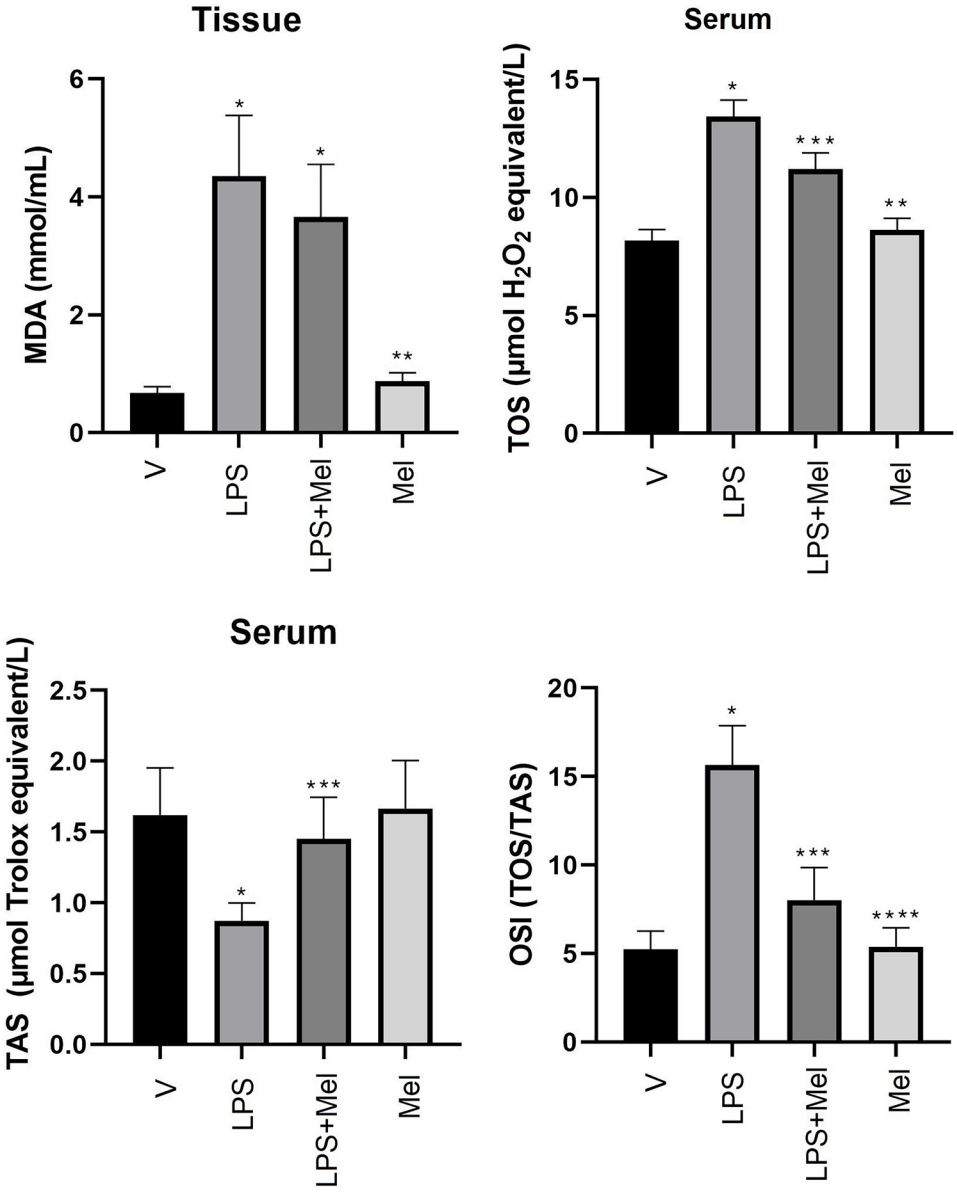

Figure 8. Mean tissue MDA, serum TOS, TAS, and TOS/TAS oxidative stress index of all groups are summarized. *$p < .001$ versus V, **$p < .001$ versus LPS + Mel, ***$p < .001$ versus LPS, ****$p < .001$ versus LPS + Mel.

Mean tissue glutathione, SOD, and CAT levels of the LPS group were lower than those of the control group ($p < .001$). Melatonin significantly restored those values ($p < .001$). Mean serum glutathione, SOD, and CAT levels of the LPS group were also lower than those of the control group ($p < .001$). Melatonin significantly restored those values too ($p < .001$) (Figure 9).

Liver function tests

Mean serum AST and ALT levels of the LPS group were higher than those of the control group ($p < .001$). Melatonin significantly restored those values ($p < .001$). The mean values of the melatonin-administered group were similar to those of the control (Figure 10).

Discussion

Although necrosis and apoptosis are thought to be the primary cell death types during sepsis, certain other uncommon cell death routes, such as necroptosis, pyroptosis, and autophagy-induced cell death, can be triggered during sepsis [13,14]. Numerous studies have shown melatonin to have hepatoprotective properties, but few have looked at how these properties and associated molecular pathways may affect liver damage brought on by sepsis. We currently investigated the effects of melatonin on sepsis-induced necrosis, apoptosis, necroptosis, proliferation, and differentiation pathways. By histopathological analysis, melatonin had no significant impact on necrosis. Melatonin also did not affect caspase-8+ cell number which was slightly increased due to LPS. By western blotting analysis, melatonin seemed effective in reducing Bax/Bcl level which was significantly increased due to LPS ($p < .001$). Melatonin prevented liver apoptosis by increasing the expression of anti-apoptotic Bcl-2 and decreased the expression of pro-apoptotic Bax. LPS induces a Bax/Bcl-related intrinsic apoptotic pathway rather than a caspase-8-related extrinsic apoptotic pathway. The best-characterized pathways of apoptosis are the intrinsic (or damage-induced, or mitochondrial) pathway and the extrinsic (or receptor-induced, or physiological) pathway [21,22]. A few studies suggest that the target of the anti-apoptotic action of melatonin may be the intrinsic pathway that Bcl and Bax molecules

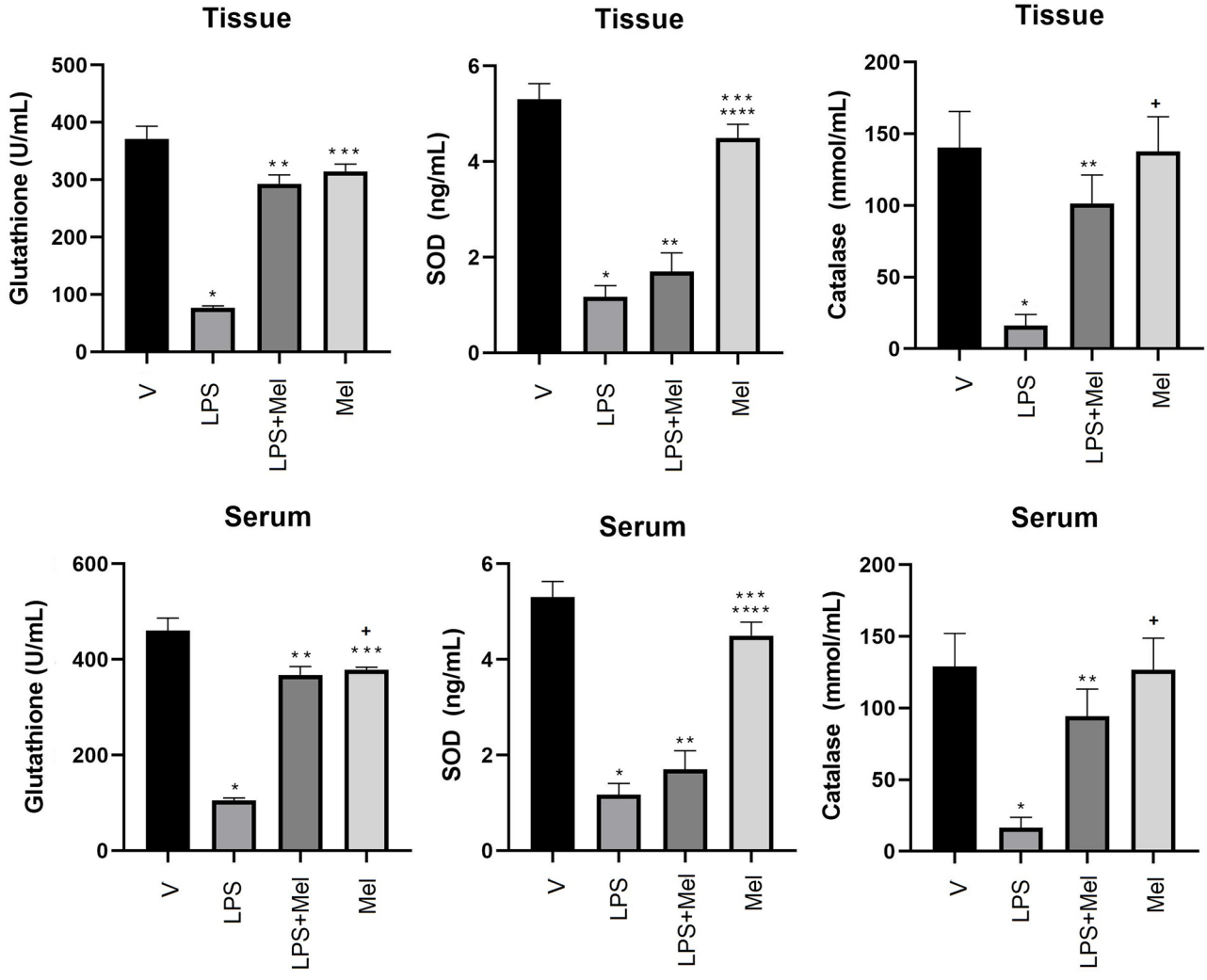

Figure 9. Tissue and serum glutathione SOD and CAT levels are summarized. *$p < .001$ versus V, **$p < .001$ versus LPS, ***$p < .005$ versus V, ****$p < .001$ versus LPS + Mel, $^{+}p < .05$ versus LPS + Mel.

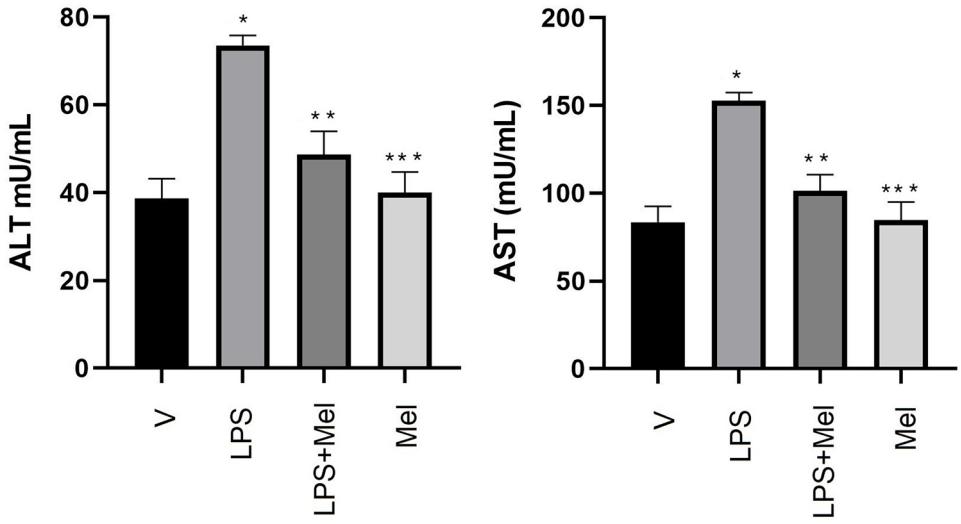

Figure 10. The mean ALT and AST levels of all groups are summarized. *$p < .001$ versus V, **$p < .001$ versus LPS, ***$p < .005$ versus LPS + Mel.

are involved in [23,24]. The intrinsic pathway which is regulated by the protein Bcl-2 begins with environmental disturbances including oxidants, Ca^{2+} rises, and pH alterations that directly or indirectly activate Bax [25].

LPS-induced oxidative stress in the organs, including the liver, was shown by tissue and serum oxidative stress markers such as TOS, TAS, glutathione, SOD, and CAT levels. MDA levels, a lipid peroxidation end product, proved oxidative stress-related cell damage. From the microscopic point of view, cell damage and tissue degeneration were already obvious in the organ samples. Melatonin reduced slightly histopathological score and tissue MDA levels. It also reduced serum TOS levels whereas it induced serum TAS levels ($p < .001$). Melatonin restored tissue and serum glutathione, SOD, and CAT levels ($p < .001$). Antioxidant and free radical scavenging power of melatonin have been known for many years. It is also detected that melatonin is a powerful antioxidant enzyme stimulant [7,26]. In the present study, we obtained some clues that LPS, in addition to necrosis and apoptosis, damages the tissue *via* necroptosis, another pathway of cell death. A type of programmed cell death known as necroptosis takes place after the receptor-interacting protein kinases RIPK1 and RIPK3 form an oligomeric complex known as the 'necrosome' [27]. The executioner protein MLKL causes lytic cell death and fast membrane permeabilization in necroptotic cells, resulting in the release of intracellular contents [28]. It was obvious that LPS-induced RIP-3 and MLKL proteins ($p < .001$) were indicators of necroptosis. Melatonin administration reduced but did not significantly affect the levels of those proteins.

Massive cytokine release, oxidative stress, and mitochondrial dysfunction are all hallmarks of sepsis' dysregulated and overwhelming inflammatory response [29]. In the present study, it was obvious that LPS-induced inflammatory reactions as well as different types of cell death. By histopathological analysis of the tissue samples stained with H&E, inflammation was observed in the degeneration area described as a necrosis area. By western blotting analysis tissue IL-1β, one of the pro-inflammatory cytokines, and NF-κB levels of the LPS group were higher than that of the vehicle group ($p < .001$). The level of NF-κB was consistent with the up-regulation of IL-1β. Inhibition of activation of NF-κB leads to attenuation of the production of pro-inflammatory cytokines and inhibits apoptosis [30]. Unfortunately, by microscopic examination and western blotting analysis, melatonin, at that dose and duration, was not found to be effective in reducing inflammatory response.

When the proper microenvironment conditions are present after any injury, the tissue will attempt to regenerate itself either by triggering the rapid division of existing cells or by stimulating stem/progenitor cells to differentiate into tissue-specific active cells. In the present study, 1 day after sepsis induction, the proliferation rate was still very low versus control ($p < .001$). Melatonin, at that dose and duration, was not competent to induce proliferation rate. OV6 immunohistochemistry revealed the same distribution pattern in all the groups. It was obvious that OV6-positive cells were not activated yet. Melatonin was not competent to induce OV6+ cells.

The results of liver function tests including serum AST and ALT levels directly give clues about the function of hepatocytes. Low AST and ALT levels in the LPS group suggest hepatocyte dysfunction, which is normal in an abnormal or damaged microenvironment. It was obvious that melatonin was highly effective in restoring hepatocyte functions ($p < .001$).

Conclusion

In this study, we provided evidence of the benefits of melatonin in terms of inhibiting the intrinsic pathway of apoptosis and tissue oxidant levels, stimulating tissue antioxidant enzyme levels, and restoring hepatocyte functions. We suggest that pre-sepsis melatonin administration might be beneficial for reducing LPS-induced hepatic damage. A single dose of melatonin administration after sepsis induction might not be sufficient for histopathological recovery. Longer-term melatonin administrations after sepsis induction may produce anti-inflammatory and anti-necrotic effects.

Author contributions

Study conception and design: M.E. and A.H.Y. Animal experiments: T.K.K. Data collection: T.K.K., F.B.K.-C., and E.R.H. Analysis and interpretation of results: M.E., B.E., and E.K. Draft manuscript preparation: M.E. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

Approval for the study was granted by Bezmialem Vakıf University Local Ethics Committee for Animal Experiments (Ethic Committee No. 2018/131).

Disclosure statement

The authors have no conflicts of interest to disclose.

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Data availability statement

All data generated or analyzed during this study are included in this published article.

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