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Determination of Melatonin Deprivation Impact on Sepsis With Acute Phase Reactants

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ABSTRACT

Background: The aim of the present study is to determine the association of melatonin hormone level on CRP, Total Antioxidant Status, Leukocyte, Procalcitonin, and Malondialdehyde, all acute phase reactants in the dark and light cycle of rats with sepsis model.

Materials and methods: In this study, 54 rats were divided into three groups. Whereas the first and third groups had a 12 h dark-light cycle, the second group was exposed to light for 24 h at 21°C-22°C for 10 d without any water and food restrictions. In the second and third groups, sepsis model was formed by cecal ligation and puncture (CLP) method at the end of 10th day, and blood samples were taken at the end of the 10th day. C-reactive protein, Malondialdehyde, Procalcitonin in the blood samples were analyzed by ELISA, and the levels of Total Antioxidant Status and leukocyte were determined by colorimetric method in the subsequent 12 and 24 h.

Results: CRP values increased in the second group rats, which were kept continuously under light and had undergone CLP, from 288.8 mg/L to 584.0 mg/L at the end of the 12 h and the end of the 24 h, approximately, two times. In rats, which were kept under 12 h of light, 12 h of darkness, and applied CLP (group 3), these values increased from 416.9 to 619.1; an increase of 1.5 times. When assessed for MDA, it was determined that the differences between Group 2 and Group 3 were more prominent between 0 h and 12 h. While the MDA values in group 2 increased from 16.53 nmol/mL at the 12 h to 17.66 nmol/mL at the 24 h. However, MDA values did not yield statistically significant changes in the third group. Changes in the in PCT values were similar to the MDA values obtained. Increase coefficient of the PCT values between 0 h and 12 h in the second group 2 was 1.26; however, in the third group, it was negligible.

Conclusions: An increase in the oxidative stress was observed in the rats that underwent CLP and melatonin deprivation via continuous 24 h light exposure for 10 d. Accordingly, deprivation of light is considered to be effective in sepsis treatment due to the increase in melatonin levels in intensive care unit patients.

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Introduction

Globally, nearly 14 people die due to sepsis every min, and this number adds up to nearly 2,00,000 people per day.[1] It is estimated that the annual death in UK rate is 30 to 50 per 1,00,000 individuals is between.[2,3]

Sepsis can be described as developing systemic inflammatory response syndrome (SIRS) in response to infection. Although numerous infections can lead to sepsis, respiratory, and circulatory system infections and intraabdominal focus are the most common types. In hospital-acquired sepsis cases, especially catheter-related infections are the most common infections.[4]

More recently, changing factors leading to sepsis have been understood better, and new factors may emerge as this is a dynamic process leading to the emergence of novel and different treatment options (such as antiendotoxin therapy).[5,6]

A total score to be generated by procalcitonin (PCT), C-reactive protein (CRP) and sepsis-related organ failure (SOFA) can be considered as a valuable estimation tool in the accurate diagnosis of sepsis.[7] Lorente *et al.* suggested that serum MDA, one of the biomarkers of oxidative stress, could provide prognostic information about the risk of mortality in sepsis. In their study with severe septic patients, the largest series providing information about the oxidative status, high levels of malondialdehyde (MDA) may represent unstable oxidant status and is associated with poor prognosis in severe sepsis patients.[8] Serum total antioxidant capacity value can be used to evaluate the actual change in antioxidant status in patients with severe sepsis and may be beneficial for treatment.[9] Chuang *et al.* reported that serum total antioxidant capacity (TAS) levels were higher in patients with severe sepsis, especially in nonsurvivors, and could reflect clinical severity.[10]

A study on rats in sepsis treatment, determined that sepsis caused increased renal vascular resistance. Some antioxidant agents used suppressed this increase and increased renal vascular resistance by removing free oxygen radicals and changing arachidonic acid metabolism.[11] Melatonin is a hormone released at dark from the pineal gland, which is a neuroendocrine organ.[12] The pineal gland, which releases the melatonin hormone, controls many physiological functions, such as regulating hormone system, preserving the effects of hypothalamus and hypophysis, stimulating the immune system, and removing free radicals.[13,14] It was stated that the melatonin level was also related to sepsis severity and mortality.[15] Although there are many factors that affect melatonin synthesis and release, light is the most important one. In other words, the day-night rhythm, light-dark cycle controls the regulation of synthesis and oscillation. This regulation system is defined as "photoneuroendocrine control." In addition to regulating the circadian rhythm in melatonin synthesis, light can suppress melatonin synthesis acutely. In particular, the effects of artificial light on the eyes during the night may result in a sudden decrease in nocturnal synthesis and release of melatonin.[16] Melatonin is thought to affect immune system cells through melatonin receptors. Melatonin receptors detected on leukocytes and lymphocytes are shown as proof of this.[17] Melatonin is a powerful free radical scavenger. With this effect, it inhibits the activity of nitric oxide synthase enzyme and shows an antioxidant effect.[18,19] Melatonin also prevents the oxidant damage caused by neutrophil activation in the tissues in the ischemia-reperfusion injury, sepsis, burn injury, and similar inflammatory diseases. The inhibitory effect of melatonin on the immune response has been suggested to be beneficial in organ transplantation because of its relevance to the antioxidant properties of the molecule. The lack of toxicity of this agent also supports the reliable use of this agent in transplantation.[20]

The aim of the present study is to determine changes caused by the antioxidant effect of melatonin on sepsis and to make a contribution to sepsis treatment. Accordingly, the effects of melatonin deficiency on leukocyte, procalcitonin, total antioxidant, C-reactive protein, and malondialdehyde levels in the serum were investigated in rats with artificial sepsis.

Materials and methods

Animal experiments of the present study were conducted in the laboratories of Selcuk University Experimental Medicine Application and Research Center (SÜDAM) (Animal Experiments Ethics Committee approval no: 2015/37). Leukocyte, Procalcitonin, Total Antioxidant levels, C-reactive protein, and Malondialdehyde analyses of the blood samples in Selcuk University Medical Faculty Medical Biochemistry Research and Application Laboratories.

Animal materials

A total of 54 adults (4-5 mo old) Wistar Albino male rats weighing 200-300 g, provided by SÜDAM, were used in the present study. Rats were randomly separated into three groups. Animals in the first g and third group were kept at 21°C-22°C under a 12 h light-dark cycle without any water and food restrictions. In order to enable melatonin deprivation, animals in the second group were kept at similar conditions, 21°C-22°C, under continuous white light (202 lux) for 24 h; likewise, without water and food restrictions (artificial pinealectomy (PLT)). Feed restriction was applied to all rats 12 h before surgeries requiring anesthesia.

Processes applied

Group 1 (SHAM; $n = 18$): For 10 d, six rats were randomly selected from rats exposed to 12 h light and 12 h darkness, their thoracic cavities were opened under anesthesia, and approximately 8-10 mL blood samples were taken from the hearts with an injector. The time at which these blood samples were taken was accepted as 0 h. The abdomens of the remaining 12 rats were opened, and the cecum was removed and relocated without puncture and ligation. After 12 h, 8-10 mL blood was collected from six randomly selected rats by the method described above. A similar situation was repeated after 24 h for the remaining six rats.

Group 2 (PLT + CLP; $n = 18$): For 10 d, six rats were randomly selected from rats exposed to 24 h light, their thoracic cavities

were opened under anesthesia and approximately 8-10 mL blood samples were taken from the hearts with an injector. The time at which these blood samples were taken was accepted as 0 h. The thoracic cavities of the remaining 12 rats were opened, and the cecum was removed and relocated after the puncture and ligation processes (CLP). After 12 h, 8-10 mL blood was collected from six randomly selected rats by the method described above. A similar situation was repeated after 24 h for the remaining six rats.

Group 3 (CLP; $n = 18$): For 10 d, six rats were randomly selected from rats exposed to 12 h light and 12 h darkness, their thoracic cavities were opened under anesthesia and approximately 8-10 mL blood samples were taken from the hearts with an injector. The time at which these blood samples were taken was accepted as 0 h. The thoracic cavities of the remaining 12 rats were opened, and the cecum was removed and relocated after the CLP operation. After 12 h, 8-10 mL blood was collected from six randomly selected rats by the method described above. After 24 h, a similar situation was repeated for the remaining six rats.

After taking the final blood samples, all rats used in the present study were terminated. Blood taken from the rats in all groups were distributed to EDTA and plain blood tubes.

Experimental design

Intraperitoneal ketamine 60 mg/kg and xylazine HCl 10 mg/kg (Ketalar and Citanest, 2%, Eczacıbaşı, Turkey) were used for general anesthesia in all surgical interventions in the study.

Cecal ligation and puncture (CLP) model was applied to form the sepsis model in rats.[21,22] Our reason to prefer the cecal ligation and puncture model was for the formation of our sepsis model from multiple microorganisms and the occurrence of a clinical presentation similar to septic shock. Blood samples taken from rats were centrifuged at 3000 revs/min for 5 min after keeping at room temperature for 30 min and then were separated into serums. They were kept at −21°C until the test was started.

Survival conditions of the rats at the end of this study are provided in Figure 1. As in Figure 1, three of the rats in Group 2 (Artificial-PLT + CLP) died at the end of 24 h. One rat died at the end of 12 h in Group 3 (CLP).

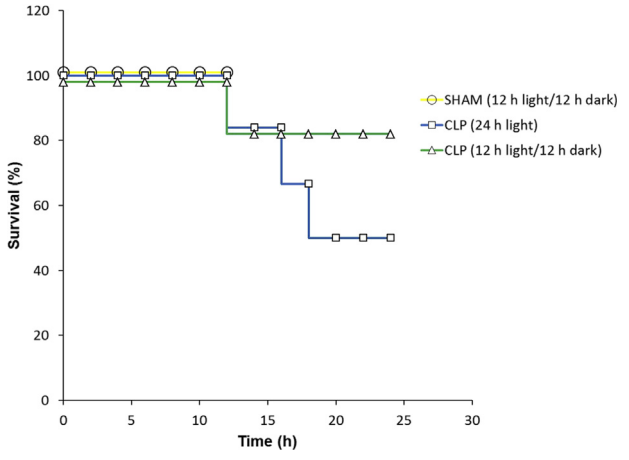

Fig. 1 – Survival results of rats at the end of the study. (Color version of figure is available online.)

Biochemical analysis

CRP, MDA, PCT designations

In line with kit study procedures, they were analyzed using Abcam(USA) brand rat commercial kits on Rayto-2100C Microplate Reader (India) device located in Selçuk University Faculty of Medicine Biochemistry Research Laboratory with Enzyme-Linked Immunosorbent Assay (ELISA) method at 450 nm in CRP 5 ng/mL-900 ng/mL, MDA 0.3 nmol/mL–65 nmol/mL, PCT 25pg/mL–7200pg/mL sensitivity rate. Units are CRP- mg/L, PKT-ng/mL, MDA-nmol/mL'.

TAS designation

Serum-separated blood samples were analyzed with the colorimetric method on the Abbott Architect C16000 (Japan) device located in Selcuk University Faculty of Medicine Biochemistry Laboratory with RellAssay brand commercial kits after calibration-control operations.[23] Results were given with mmol Trolox Equivalent/L unit. TOS analysis could not be done as the blood sample was inadequate.

Leukocyte designations (WBC)

Samples taken in blood tubes with EDTA were analyzed on the Abbott CELL-DYN 3700 System (USA) device located in Selcuk University Faculty of Medicine Biochemistry Laboratory. Results were given with K/mm^3 unit.

Statistical analyses

Kolmogorov–Smirnov test was used to determine whether the data had a normal distribution. As none of our data showed normal distribution, nonparametric tests were employed. While difference among the groups was detected with Kruskal–Wallis Variance analysis, paired comparisons of different parameters were made with Bonferroni corrected Mann–Whitney U-test. Wilcoxon Signed-Rank test was applied for evaluating the difference among the groups.

Results

Biochemical results

Change in leukocyte, CRP, MDA, PCT and TAS values with 12/24 h of light application

Definitive statistics of leukocyte, CRP, MDA, PCT and TAS values for light-time combinations are provided in Figures 2-6.

Changes were observed in starting values of study parameters compared to the values acquired from rats kept continuously under light for 24 h and those kept with a 12 h light-dark cycle; however, these changes were statistically insignificant.

Statistically significant results were found between the first with leukocytosis occurrence and the second and third groups with leukopenia occurrence based on leukocyte results at the 12 h (p:0.008, p:0.012 in order). A significant difference was also found in CRP values at 12 and 24 h among the first

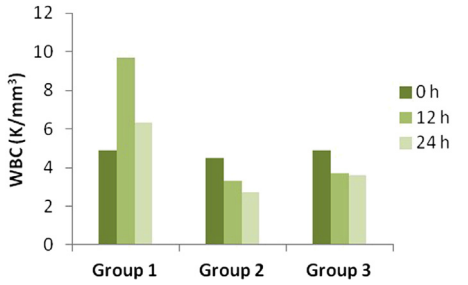

Fig. 2 – Light-time combinations for Leukocyte (WBC) values of study groups in rats. a: Difference between Group 1 and Group 2 for WBC on 12 h p:0.008; b: Difference between Group 1 and Group 3 for WBC on 12 h p:0.012. (statistical significance $P < 0.05$). (Color version of figure is available online.)

and third groups (p:0.018, p:0.038 in order). A significant difference was found in TAS values at 24 h among the rats in the first and third groups (p:0.022).

In line with the literature, leukocytosis increased in 12 h in rats kept under 12 h in light +12 h in the dark, and a decrease toward the normal value was observed at 24 h in our study. Sepsis-related leukopenia occurred at 12 and 24 h in rats in the second and third groups.

In the present study, the rapid increase in PCT level in the first 12 h could be attributed to postoperative stress in rats, which were under 12 h light-dark cycle and to the applied opening-closure to the abdominal region. In rats, which were kept continuously for 24 h in the light and underwent cecal administration, a rapid increase in PCT levels was observed in the first 12 h due to infection.

On evaluating the results of this study, an increase was observed in MDA values in the first 12 h in all groups due to oxidative stress.

It was also observed that CRP values increased in the first 12 h in the second and third groups due to the sepsis model formed with a steady increase in 24 h. The change toward an increase in CRP value demonstrated that the sepsis model has functioned in the rats.

TAS values were at a higher level compared to the other groups due to the high oxidative stress in the second group.

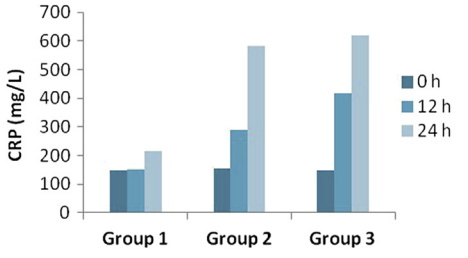

Fig. 3 – Light-time combinations for C-reactive protein (CRP) values of study groups in rats. c: Difference between Group 1 and Group 3 for CRP on 12 h p:0.018; d: Difference between Group 1 and Group 3 for CRP on 24 h p:0.038. (statistical significance $P < 0.05$). (Color version of figure is available online.)

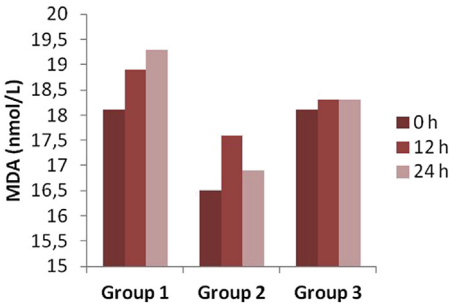

Fig. 4 – Light-time combinations for Malondialdehyde (MDA) values of study groups in rats. (Color version of figure is available online.)

Time-related decrease of TAS values in the samples demonstrate that antioxidants are consumed in infection/sepsis.

The difference in WBC values 0 h and 12 h are statistically significant based on the evaluation of the difference within the group via Wilcoxon Signed-Rank test (p:0.028). Based on the statistical evaluation made, it was found that this increase in the third group was statistically significant between 0 h and 12 h (p:0.043). It was observed that the MDA level increased with increasing SOR levels at the end of 24 h in the first group. No statistical significance was observed in time-related increase. There was a statistically significant increase in PCT values in the first and second groups in 12 h compared to 0 h (p:0.043, p:0.046 in order). A statistical significance was found between 0 h and 24 h values in the first group (p:0.046). Decreases in TAS values at 0 h and 12 h of the third group were found to be statistically significant as well (p:0.043).

Discussion

In the present study, in order to determine the antioxidant effect of melatonin on sepsis, the rats were exposed to light for 24 h and to a 12 h light-dark cycle for a period of 10 d. The effects of Melatonin deficiency on leukocyte (WBC), procalcitonin, total antioxidant, C-reactive protein and malonaldehyde levels in serum were investigated in sepsis-created rats. Leukocyte acts as diagnostic markers for sepsis and septic shock, but the ability of these parameters to predict sepsis in the early stage of the disease is unknown.[24] In the present

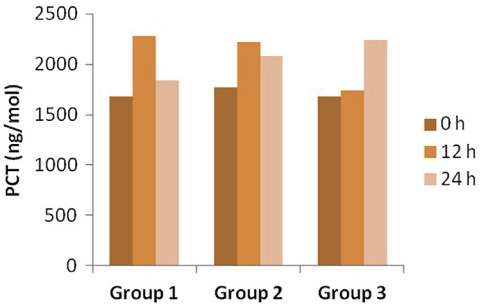

Fig. 5 – Light-time combinations for Procalcitonin (PCT) values of study groups in rats. (Color version of figure is available online.)

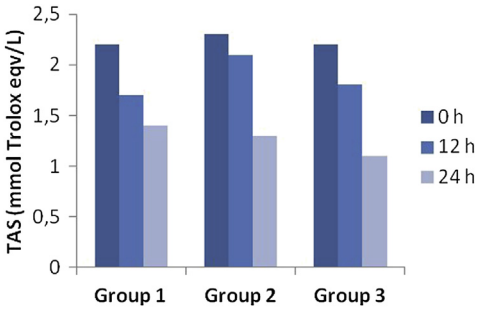

Fig. 6 – Light-time combinations for Total Antioxidant Status (TAS) values of study groups in rats. e: Difference between Group 1 and Group 3 for TAS on 24 h p:0.022 (statistical significance $P < 0.05$). (Color version of figure is available online.)

study, leukocyte changes in the detection of sepsis and melatonin deficiency created by darkness are determined. A previous study reported that total leukocyte count measurements made in the first 24 h are useful for early diagnosis of sepsis.[25] Shrum *et al.* stated that a histological examination of the tissues demonstrated different degrees of pathology in various organs at 24 h after fecal-induced peritonitis (FIP). In the lung, they observed mild edema in the alveolar spaces and leukocyte accumulation in the peripheries of pulmonary arterioles.[26]

CRP is a common biomarker to detect infection and inflammation. As CRP levels increase significantly during acute inflammation compared to other acute phase reactants, CRP testing has been in practice for a long time to detect the presence of systemic inflammation, infection, or sepsis.[27] The increase in CRP synthesis directly indicates the intensity of the inflammatory process. Conditions, such as anastomosis leakage, wound infection, tissue ischemia, and necrosis, mobilize CRP synthesis. In septic patients, a decrease in CRP levels indicates a higher survival rate or dissolution in sepsis. Ersoy *et al.* reported that melatonin application decreased C-reactive protein levels in the CLP applied rats, and the decrease in CRP levels in these rats confirmed the anti-inflammatory role of melatonin during sepsis.[28] CRP is mainly synthesized with IL-6 stimulus in response to inflammatory or fever conditions such as infection, injury, surgery, trauma, tumor, and tissue necrosis. CRP blood levels increase within 4-6 h, reach their highest level in 24-36 h (negative predictive value approaches 100%) with a half-life of 19 h. However, in the presence of organ (liver) failure, the decrease of its synthesis is delayed. A low CRP level does not exclude bacterial infection. CRP value can be found as negative in the first 12 h at the onset of a disease. Thus, if a bacterial infection is suspected clinically, a series of CRP measurements should be made.[29] Increased CRP level in bacterial infections does not show the etiology of the infection, although it is parallel to tissue damage.[30] Povoa *et al.* reported that daily CRP measurement was useful for sepsis diagnosis and was more sensitive than markers such as WBC used today.[31] Cutando Soriano *et al.* applied topical melatonin on diabetes patients once a day for 20 d. As a result, the researchers determined a significant decrease in CRP levels of diabetes patients who were applied melatonin compared to those who were not applied melatonin.[32] Steel and Whitehead reported that the CRP level starts to increase in 4-6 h after the onset of inflammation and reaches its highest value after 24-48 h[33] EL-Gendy *et al.* stated that 20 mg melatonin in addition to the administration of antibiotics in 40 neonates with proven sepsis under clinical and laboratory conditions created a more positive effect on CRP and serum parameters compared to patients treated only with antibiotics, and thus, made a positive contribution on sepsis treatment.[34] In the present study, the increase in CRP values has demonstrated the presence of sepsis in rats with CLP, with an increase with time.

Another marker is theMDA indicator of increased oxidative stress. It has an active role as a marker in diseases with an increase in lipid peroxidation. Poly unsaturated fatty acids peroxidation is the basic resource of MDA in all biological activities.[35] As MDA is a lipid peroxidation product, it was studied as an oxidative stress marker in many pathological cases in which peroxidation was considered to increase. Studies have shown that MDA is a parameter causing the onset of cancer in many cancer types and is also a marker in lipid peroxidation. Stomach cancer, breast cancer, chronic myelocytic leukemia, and cervical cancer can be named among cancers with a high malondialdehyde level.[36,37] In their research, Şener *et al.* stated that sepsis increased MDA values and oxidative mechanisms played a role in the sepsis-caused tissue destruction. In the same study, it was also reported that melatonin created protective effects against sepsis-caused oxidative organ damage.[38] As seen in the present study too, time-related proportional increases in MDA values were higher in conditions with melatonin deprivation conditions and less in those without. This finding could be attributed to the protective effect created by melatonin against oxidative damages as mentioned in the study by Şener *et al.*[38]

A further marker is Procalcitonin (PCT). Like CRP, it is employed to determine risk factors of septic shock.[39] Redl *et al.* determined in a study on monkeys, animals, as well as in humans, procalcitonin is the primary marker of bacterial-induced sepsis.[40] Park *et al.* stated that the increase of procalcitonin is a quite effective early diagnosis marker of neonatal infection but not as reliable as C-reactive protein.[41] It has been shown that the administration of procalcitonin to septic animals greatly increases mortality, and various toxic effects of procalcitonin have been explained by *in vitro* experimental studies. Antibodies have been developed to neutralize the deleterious effects of procalcitonin, and their use significantly reduces the symptomatology and mortality of animals with high virulent sepsis, similar to those occurring in humans.[42] Liu *et al.* investigated the role of PCT in distinguishing patients with sepsis and systemic inflammatory response syndrome and the provision of the control of these diseases. As a result of this research, it was observed that procalcitonin and interleukin-6 levels demonstrated a distinguishable level of increase compared to other inflammatory reaction criteria as the diseases progress.[43] Sharman and Bondy reported that sepsis was effective in increasing of procalcitonin level but did not have any relation with the melatonin level.[44] Park *et al.* stated that the increase of procalcitonin is a quite effective early diagnosis marker of neonatal infection but was not as reliable as CRP.[41] In the present study, high procalcitonin levels at the end of 24 hr in all groups that have undergone CLP demonstrates the

presence of sepsis as seen in increased leukocyte and CRP values. Moreover, the procalcitonin level in the serums of the rats with melatonin deprivation did not demonstrate a significant change compared to the levels in the serums of rats without melatonin deprivation. This shows us that the procalcitonin level possibly cannot be related to melatonin as reported by Sharman *et al.*[44]

The findings of the present study show that melatonin deprivation leads to oxidative stress in rats, and therefore, TAS values were higher in rats with melatonin deprivation. The decrease in TAS values in the samples over time was thought to be consumed by using antioxidants in infection/sepsis. Mammals have many antioxidant systems to cope with oxidative stress of vital importance for the smooth operation of the systems.[45] Benot *et al.* detected that, compared to control rats kept under dark, the serum TAS values significantly decreased when the rats were continuously kept under light between the 20.00 and 05.00 h. Since the administration of exogenous melatonin also increases the TAS value in rat serum, the results suggest that melatonin may be appropriate in terms of incorporation into the antioxidant capacity of rat serum.[46] Using the total peroxyl radical trapping method, Pascual *et al.* determined that plasma antioxidant capacity was low in septic patients and was higher in septic shock patients compared to the control group. Even though the total plasma antioxidant capacity decreased from normal levels in septic patients in their study, the researchers stated that the increase in some oxidants contributed to an increasing total antioxidant capacity in septic shock patients.[47] Although there is a partial contradiction between the findings of the present study and the results of Benot *et al.*,[46] they are in line with the findings acquired by Pascual *et al.*[47]

Conclusion

In the present study, benefiting from the outcomes of previous research on the effects of melatonin on the immune system, changes in WBC, MDA, PCT, CRP, and TAS values in rats with a formed septic shock are examined.

On analyzing the results, it could be concluded that sepsis model formation was successful. The results indicate during the treatment of sepsis in intensive care units, a patients' treatment could be enhanced by increasing the melatonin level through a very easy procedure, that is by switching of the lights or bringing in a 12 h light-dark cycle into the *sterile, inhumane, and loud* intensive care units.

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Authors' contributions: H.F.A. contributed for manuscript preparation and manuscript editing. T.S. was responsible for concept design and acquisition of data. H.V. was responsible for concept design and manuscript editing. H.F.A. contributed for concept design, manuscript preparation, and editing manuscript. T.S. contributed for concept design, acquisition of data, manuscript preparation, and editing manuscript.

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Disclosure

The authors do not pursue any gain in this study.

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