ORIGINAL ARTICLE

An Adjuvant Treatment of Melatonin Prevented the Elevation of Leukocyte Count and the Decrease of Platelet Count in Wistar Rats Endotoxicosis Model

SATRIO ADI WICAKSONO[1], DINI AYU HARISIANI[2], RIZQI INDAH RIANI[3], SULISTIYATI BAYU UTAMI[4]
[1]*Head of Department of Anesthesiology and Intensive Therapy. Faculty of Medicine. Diponegoro University. Semarang. Indonesia.*
[2,3]*Faculty of Medicine. Diponegoro University. Semarang. Indonesia.*
[4]*Head of Study Program. Department of Cardiology and Vascular Medicine. Faculty of Medicine. Diponegoro University*
Correspondence to: Satrio Adi Wicaksono, E-mail: drsaw11@yahoo.com, Telephone: +62 024-76928010

ABSTRACT

Background:: Sepsis causes systemic inflammatory response syndrome (SIRS) that effects on coagulation cascade, tissue hypoxia, leukocyte and the platelet count. Melatonin is thought to ameliorate immune response, cytoprotective process, and resist bacterial, viral, and parasites infections through immunomodulations and antioxidant activities. Melatonin decreases the levels of inflammatory cytokines, oxidative stress, and mitochondrial dysfunction, however its role for sepsis therapy still need further investigation.
Aim: To observe the effect of melatonin with and without Polymyxin B on the leukocyte and platelet count in Wistar rats endotoxicosis model.
Methods: This was animal experimental study with randomized control design. There were twenty four male Wistar rats that were intraperitoneally injected with 2 mg lipopolysaccharide (LPS) per 200 grams rats and randomly divided into four groups, i.e. control group receiving placebo, melatonin only group, Polymyxin B only group, and combination group (receiving melatonin and Polymyxin B). The effects of oral melatonin and Polymyxin B treatments on leukocyte and platelet count were investigated from the retro-orbital blood vessel sampling in baseline (B), 6 hours after intraperitoneally LPS injection (T0), 1 hour (T1) and 2 hours after melatonin administration (T2). The mean differences of leukocyte and platelet count were analyzed with Student's *t*-test.
Results: There were no significant differences in leukocyte and platelet count between control group, melatonin only group, Polymyxin B only group, and combination group in B and T0. In T1, there were significantly lower leukocyte count in melatonin only group (11,400 ± 3,200/mm^3) or in Polymyxin B only group (10,300±1,200/mm^3) or in combination group (9,900 ± 1,200/mm^3) compared to control group (13,900±1,300/mm^3, p<0.05). In T2, there were significantly lower leukocyte count in melatonin group (12,500±2,700/mm^3) or in Polymyxin B only group (10,400±1,500/mm^3) or in combination group (9,850±1,100/mm^3) compared to control group (15,700±1,500/mm^3, p<0.05). In T1, there were significantly higher platelet count in melatonin only group (167,500±71,500/mm^3) or in Polymyxin B only group (175,900±72,300/mm^3) or in combination group (178,500±52,100/mm^3) compared to control group (148,100±62,800/mm^3, p<0.05). In T2, there were significantly higher pletelet count in melatonin group (149,900 ± 61,600/mm^3) or in Polymyxin B only group (158,600 ± 51,500/mm^3) or in combination group (162,500 ± 51,100/mm^3) compared to control group (125,600 ± 51,200/mm^3, p<0.05).
Conclusion: Melatonin and Polymyxin B administration, alone or in combination, could prevent the elevation of leukocyte count and the decreased of platelet count in Wistar rats endotoxicosis model. Therefore, if confirmed by further research, melatonin is promising in the management of endotoxicosis.
Keywords: Endotoxicosis, leukocyte count, platelet count, melatonin, lipopolysaccharide

INTRODUCTION

Sepsis is a serious clinical syndrome that occurs due to the body's excessive response to stimulation of microorganism products. Symptoms occured are including fever, tachycardia, tachypnea, hypotension, leukocytosis or leukopenia, and sometimes multiple organ dysfunction or failure (MOF) associated with impaired circulation[1].

Sepsis impact on high annual economic burden[2,3]. Some factors correlated with sepsis in developing countries were low socio-economic status, poor-hygienic environment and other concomitants diseases, such as diabetes mellitus, immunosuppression, malnutrition, and cancer[3,4]

Sepsis can be divided into severe sepsis and septic shock which is an important cause of death. Sepsis is a significant health problem since it is one of the frequent causes of mortality in the non-coronary intensive care unit (ICU)[1,3,4,5]. The incidences of sepsis and septic shock were abundant in the United States, which were 10,319,418 cases from 750 million in-hospital care during 22 year study period.[5] The mortality rate of sepsis was 215,000 (9.3%) of all deaths in the United States in 1995.[2] The study group in Management of Severe Sepsis in Asian Intensive Care Units (MOSAIC) showed that in-hospital mortality rate due to sepsis in 16 Asian countries in 2009 was 44.5%[6]. Severe sepsis in southeast Asia from 2013 to 2015 was 194(28%) of 731 children and 546(68%) of 804 adults, and was correlated with increased mortality[7].

Sepsis might induce circulating cytokines storm causing inflammation storm, metabolic disorders, coagulation disturbance, multiple organ failure and mortality[8,9]. Sepsis is often followed by some complications that will worsen the prognosis, such as Disseminated Intravascular Coagulation (DIC)[10]. In US, there was 18,000 cases of DIC in 1994. DIC was occured in 30-50% patients with sepsis, and in 1% of patients in-hospital care. The mortality rate depends on the level of disease severity. In

overall, mortality rate-related DIC in children with sepsis is 13-40%. While in developing countries, this number could be more than 90%[11].

Future sepsis management may develop along with a better understanding of the pathophysiology of sepsis, such as biomarkers that are capable to assess the diagnosis and prognosis in sepsis. The faster we can diagnose and manage severe sepsis, the better the intervention and the prognosis[12,13,14]. These biomarkers are procalcitonin, C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukins (ILs). Meanwhile, the gold standard in diagnosing sepsis is microbiological culture that will need longer time[12,14].

The increased expression of proinflammatory cytokines, such as TNF-α and ILs, was considered to associate with activated nuclear factor kappa-B (NF-κB)[8,15] and Toll-like receptor (TLR)[15]. However, these biomarkers have some limitations, such as expensive, less availability, and longer laboratory examination[12,16]. Thus, it is needed an easier examination that were more feasible[13,16] and inexpensive,[17] such as hematologic findings.

Patients with sepsis are often showing leukocytosis or leukopenia and thrombocytopenia in their hematologic findings. Leukocytosis or leukopenia in patients with sepsis was caused by proinflammatory response to infection.[11] There was a higher leukocytes in non-survivors group compared to survivors group in day 1–3[18]. In severe sepsis or septic shock, disseminated intravascular coagulation (DIC) may be occured. It is a condition in which blood clots form throughout the body, blocking small vasculatures. As clotting factors and platelets are used up, thrombocytopenia, low fibrinogen, high INR, high D-dimer, and bleeding may occur.[10] Leukocyte count and platelet count might be used as simple parameters in progressivity and prognosis of sepsis, due can predict the existence of DIC[18,19].

Melatonin (N-acetyl-5-methoxytryptamine) is a multi-functional endogen indolamine and serotonin-derived neurohormone molecule secreted by the pineal gland[20]. Melatonin acts in regulating sleep patterns or circadian rhythm, immunoregulation, immunomodulation, mitochondrial protection function, mood, reproduction, as well as a potent antioxidant, anti-inflammatory, sedative, analgesic, and chronobiotic effects[20,21]. The usefulness of melatonin has been discussed in several clinical applications such as critical care, pain management, and perioperative management[20]. Melatonin treatment is promising in ischemic or reperfusion state[20].

Polymyxin B is a cationic peptide antibiotic that can neutralize endotoxin. Polymyxin B with the endotoxin neutralizing ability might be used as a non-toxic therapeutic agent to encounter endotoxicosis[22]. Harm et al showed in vitro inactivation of endotoxins by polymyxin B[23]. Amongst several approaches in combating endotoxic shock, peptide mediated neutralization of LPS seems to be the most promising.[22] Inactivation of endotoxins by polymyxin B infusion may be used to resolve the necessity for endotoxin elimination in treatment of sepsis[23].

Previous studies showed that melatonin and 6-hydroxy-melatonin have been developed as sepsis therapy,[20, 24-26] that act as anti-inflammatory, antioxidant, and taken together as immune system enhancer[24-26]. Melatonin could reduce inflammatory cytokines levels, oxidative stress and mitokondrial dysfunction[24]. This study aimed to examine the effects of melatonin with and without Polymyxin B on leukocyte count and platelet count in the Wistar rats model of endotoxicosis, since leukocyte count and platelet count might be used as simple parameters for progressivity and prognosis of sepsis[27].

METHODS

This was an animal experimental study with randomized control group design. The inclusion criterias were male Wistar rats aged 2-3 months, body weight 150-300 grams, no physical abnormality and looked active during adaptation periode. In this study, an appropriate endotoxicosis model was created in rats through intraperitoneal injection of 2 mg lipopolysaccharide (LPS) suspension per 200 grams rats. The effects of oral melatonin and Polymyxin B treatments on leukocyte count and platelet count were investigated. Ethical clearance for animal conduct have been recieved from ethical committee.

The study samples were twenty four (24) male wistar rats which consist of six (6) male wistar rats for each group. All rats were adapted for seven days and fed with standard food. On the day-8, all samples were intraperitoneally injected with 2 mg LPS per 200 grams rats and then divided into four groups through simple random sampling. All rats were labeled by hidden number, so the blood sample taken could be numbered by the same hidden numbers.

Group 1 or control group was orally administered with placebo only (aquadest) (*n*=6). Group 2 was group with orally administered 4 mg melatonin treatment only per 200 grams rats via oral sonde (*n*=6), group 3 was group with Polymyxin B treatment only (*n*=6), and group 4 was group with combination of melatonin and Polymyxin B with treatment (*n*=6).

Blood samplings were taken via retro-orbital vein to measure the leukocyte count and platelet count in one hour before LPS injection (baseline) (B), six hours after LPS injection (T0), one hour after melatonin administration (T1) and two hours after melatonin administration (T2).

Leukocyte count were measured automatic method and confirmed with manual leukocyte counting method. The manual leukocyte counting method was performed with a hemocytometer using a standard microscope system and was confirmed with a blood smear sample.[28] The specimens were viewed directly through the microscope's eyepiece or captured into image files. For a blood smear sample, the monolayer regions were mechanically scanned for counting the total number of leukocyte, while for a hemocytometer, the gridded area was scanned for the counting purpose. Platelet counts were performed with an automated hematology analyzer and confirmed by manual platelet count with a hemocytometer[28].

Endotoxins lipopolysaccharides (LPS) were from Escherichia coli O55:B5 (L2880, Sigma-Aldrich, Darmstadt, Germany).[23] Polymyxin B (X-Gen Pharmaceuticals, Inc. Big Flats, NY) intravenous injection dose was 100 ng/ml as previously reported in in vitro inactivation of endotoxins by Polymyxin B[23] Melatonin treatment was using melatonin

powder (Melatonin M5250, Sigma-Aldrich, Darmstadt, Germany).

Those data were tested for normality with Saphiro-Wilk test. Leukocyte count and platelet count were compared within group using a one-way variance analysis (ANOVA). The means were compared using a parametric Student's *t*-test, including paired *t*-test and independent *t*-test. They will be tested with non-parametric Mann-Whitney Rank Test, if their distribution were abnormal. Data were analyzed with computer program. The *p*-value of less than 0.05 is considered to be statistically significant.

RESULTS

Effect of Melatonin in Leukocyte Count: There were no significant differences in the leukocyte count between the control group, melatonin only group, Polymyxin B only group, and combination group in baseline (B), respectively (8,500 ± 1,700 /mm^3; 8,400 ± 2,100 /mm^3; 8,650 ± 1,800 /mm^3; 8,550 ± 1,900 /mm^3, p>0.05) (table 1, figure 1).

There were significant differences in the leukocyte count between in six hours after LPS injection (T0) in comparison with baseline (B) in each groups (10,250±1,900/mm^3 vs 8,500±1,700/mm^3 in control group; 10,150±1,700/mm^3 vs 8,400±2,100/mm^3 in melatonin only group; 10,000±1,300/mm^3 vs 8,650±1,800/mm^3 in Polymyxin only group; and 10,100±1,900/mm^3 vs 8,550±1,900/mm^3 (p<0.05). These showed that the leukocyte count were higher in the T0 in comparison with baseline (table 1, figure 1).

In six hours after LPS injection (T0), there were no significant differences in the leukocyte count between control group and melatonin only group (10,250±1,900/mm^3 vs 10,150 ± 1,700/mm^3, p=0.459), between control group and Polymyxin B only group (10,250±1,900/mm^3 vs 10,000 ± 1,300/mm^3, p=0.483), and between control group and group with combination therapy (10,250 ± 1,900/mm^3 vs 10,100 ± 1,900/mm^3, p=0.467). In T0, there were also no significant differences of leukocyte count between group with melatonin only and group with Polymyxin B only (10,150 ± 1,700/mm^3 vs 10,000 ± 1,300/mm^3, p=0.436); between group with melatonin only and group with combination therapy (10,150±1,700/mm^3 vs 10,100±1,900/mm^3, p=0.477); and between group with Polymyxin B only and group with combination therapy (10,000±1,300/mm^3 vs 10,100±1,900/mm^3, p=0.754) (table 1, figure 1).

In 1 hour after treatment (T1), there were significant higher levels of leukocyte count in control group in comparison with melatonin only group (13,900 ± 1,300/mm^3 vs 11,400 ± 3,200/mm^3, p=0.041), between control and Polymyxin B only group (13,900 ± 1,300/mm^3 vs 10,300 ± 1,200/mm^3, p=0.038), and between control and group with combination therapy (13,900 ± 1,300/mm^3 vs 9,900 ± 1,200/mm^3, p=0.035) (table 1, figure 1).

In T1, there were no significant differences of leukocyte count between melatonin only group and Polymyxin B only group (11,400 ± 3,200/mm^3 vs 10,300 ± 1,200/mm^3, p=0.471); between group with melatonin only and group with combination therapy (11,400 ± 3,200/mm^3 vs 9,900 ± 1,200/mm^3, p=0.465); and between group with Polymyxin B only and group with combination therapy (10,300±1,200/mm^3 vs 9,900±1,200/mm^3, p=0.643) (table 1, figure 1).

In 2 hours after treatment (T2), there were higher levels of leukocyte count between the control to the melatonin only group (15,700±1,500/mm^3 vs 12,500± 2,700/mm^3, p=0.034), between control to the Polymyxin B only group (15,700±1,500/mm^3 vs 10,400±1,500/mm^3, p=0.031), and between control to the combination group (15,700±1,500/mm^3 vs 9,850±1,100/mm^3, p=0.028) (table 1, figure 1).

In T2, there were no significant differences of leukocyte count between group with melatonin only and Polymyxin B only group (12,500 ± 2,700/mm^3 vs 10,400 ± 1,500/mm^3, p=0.427); and between group with Polymyxin B treatment only and group with combination therapy (10,400 ± 1,500/mm^3 vs 9,850 ± 1,100/mm^3, p=0.563). Meanwhile, there was a significant difference between group with melatonin only and group with combination therapy (12,500 ± 2,700/mm^3 vs 9,850 ± 1,100/mm^3, p=0.043) (table 1, figure 1).

There were significant increases in leukocyte count in the control group after T2 in comparison to T0 (15,700 ± 1,500/mm^3 vs 10,250 ± 1,900/mm^3, p=0.017) and after T2 in comparison to T1 (15,700 ± 1,500/mm^3 vs 13,900 ± 1,300/mm^3, p=0.041), as well as between T1 and T0 (13,900 ± 1,300/mm^3 vs 10,250 ± 1,900/mm^3, p=0.037) with paired *t*-test. Whereas in the group with melatonin only, there were no significant increases in leukocyte count after T1 (11,400 ± 3,200/mm^3 vs 10,150 ± 1,700/mm^3, p=0.459) and T2 (12,500 ± 2,700/mm^3 vs 10,150 ± 1,700/mm^3, p=0.418) in comparison to T0, as well as after T2 in comparison to T1 (12,500 ± 2,700/mm^3 vs 11,400 ± 3,200/mm^3, p=0.434) (table 1, figure 1). These showed that the leukocyte count increased during the progression of endotoxicosis in control group, while it was not significantly increased in group with melatonin only. Melatonin alone might prevent the increase of leukocyte count after LPS injection.

There were no significant increases in leukocyte count in the group with Polymyxin B only between T2 and T0 (10,400 ± 1,500/mm^3 vs 10,000 ± 1,300/mm^3, p=0.456), between T2 and T1 (10,400 ± 1,500/mm^3 vs 10,300 ± 1,200/mm^3, p=0.437), and between T1 and T0 (10,300 ± 1,200/mm^3 vs 10,000 ± 1,300/mm^3, p=0.456) with paired *t*-test (table 1, figure 1). There were no significant increases in leukocyte count in the group with combination therapy between T2 and T0 (9,850 ± 1,100/mm^3 vs 10,100 ± 1,900/mm^3, p=0.455), between T2 and T1 (9,850±1,100/mm^3 vs 9,900 ± 1,200/mm^3, p=0.458), and between T1 and T0 (9,900 ± 1,200/mm^3 vs 10,100±1,900/mm^3, p=0.453) with paired *t*-test (table 1, figure 1). These showed that the leukocyte count increased during the progression of endotoxicosis in control group, while they were not significantly increased in group with Polymyxin B only and in group with combination therapy. Polymyxin B and melatonin might prevent the increase of leukocyte count after LPS injection.

Effect of Melatonin in Platelet Count: In baseline (B), there were no significant differences in platelet count between control group and melatonin treatment only group (295,500 ± 52,700/mm^3 vs 294,700 ± 62,100/mm^3, p=0.485); between control and Polymyxin B treatment only

group (295,500 ± 52,700/mm^3 vs 289,500 ± 62,500/mm^3, p=0.465); and between control and group with combination therapy (295,500 ± 52,700/mm^3 vs 278,100 ± 71,700/mm^3, p=0.472). There were no significant differences in platelet count between melatonin treatment only group and Polymyxin B only group (294,700 ± 62,100/mm^3 vs 289,500 ± 62,500/mm^3, p=0.481); between melatonin treatment only group and group with combination therapy (294,700 ± 62,100/mm^3 vs 278,100 ± 71,700/mm^3, p=0.435); and between group with Polymyxin B treatment only and group with combination therapy in baseline (B) (289,500 ± 62,500/mm^3 vs 278,100 ± 71,700/mm^3, p=0.438) with independent t-test (table 2, figure 2).

There were significant decreases in platelet count between six hours after LPS injection (T0) in comparison with baseline (B) in each groups (193,800 ± 51,600/mm^3 vs 295,500 ± 52,700/mm^3 in control group; 199,700 ± 92,500/mm^3 vs 294,700 ± 62,100/mm^3 in melatonin only group; 195,200 ± 91,300/mm^3 vs 289,500 ± 62,500/mm^3 in Polymyxin B only group; 199,500 ± 62,600/mm^3 vs 278,100 ± 71,700/mm^3 in combination group) (p<0.05). These showed that LPS had significant effects on the decreased of platelet count in the T0 compared to baseline (table 2, figure 2).

In six hours after LPS injection (T0), there were no significant difference in platelet count between control group and melatonin treatment only group (193,800± 51,600/mm^3 vs 199,700±92,500/mm^3, p=0.278); between control group and Polymyxin B group (193,800 ± 51,600/mm^3 vs 195,200±91,300/mm^3, p=0.264); and between control group and group with combination therapy (193,800±51,600/mm^3 vs 199,500±62,600/mm^3, p=0.263). There were also no significant differences in platelet count between melatonin treatment only group and Polymyxin B only group (199,700 ± 92,500/mm^3 vs 195,200 ± 91,300/mm^3, p=0.463); between melatonin only group and group with combination therapy in T0 (199,700 ± 92,500/mm^3 vs 199,500±62,600/mm^3, p=0.378); and between group with Polymyxin B treatment only and group with combination therapy (195,200±91,300/mm^3 vs 199,500±62,600/mm^3, p=0.276) with independent t-test (table 2, figure 2).

In 1 hour after treatment (T1), there were significantly lower platelet count in control group compared to melatonin only group (148,100 ± 62,800/mm^3 vs 167,500 ± 71,500/mm^3, p=0.049); in control group compared to Polymyxin B group (148,100 ± 62,800/mm^3 vs 175,900 ± 72,300/mm^3, p=0.045); and in control group compared to group with combination therapy (148,100 ± 62,800/mm^3 vs 178,500 ± 52,100/mm^3, p=0.041) (table 2, figure 2).

In T1, there were no significant differences between melatonin only group and Polymyxin B treatment only (167,500 ± 71,500/mm^3 vs 175,900 ± 72,300/mm^3, p=0.438); between melatonin group and group with combination therapy (167,500 ± 71,500/mm^3 vs 178,500 ± 52,100/mm^3, p=0.348); and between group with Polymyxin B treatment only and group with combination therapy (175,900 ± 72,300/mm^3 vs 178,500 ± 52,100/mm^3, p=0.428) with independent t-test (table 2, figure 2).

In 2 hours after treatment (T2), there were lower platelet count in control group compared to melatonin only group (125,600 ± 51,200/mm^3 vs 149,900 ± 61,600/mm^3, p=0.047), in control group compared to Polymyxin B only group (125,600 ± 51,200/mm^3 vs 158,600 ± 51,500/mm^3, p=0.045), and in control group compared to group with combination therapy (125,600 ± 51,200/mm^3 vs 162,500 ± 51,100/mm^3, p=0.032) (table 2, figure 2).

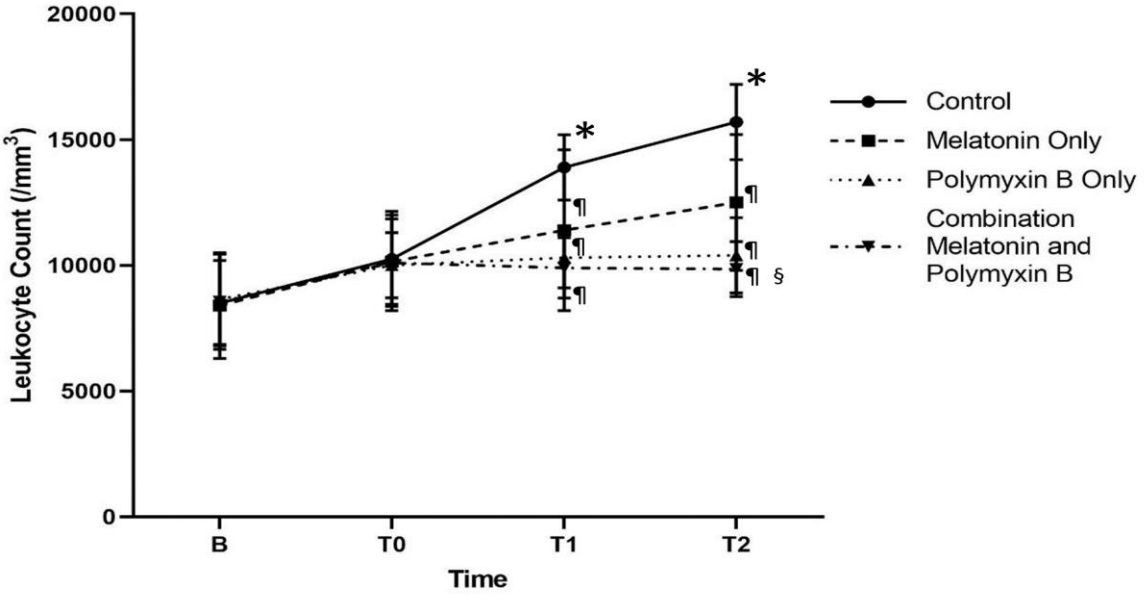
In T2, there were no significant differences of platelet count between melatonin only group and Polymyxin B only group (149,900±61,600/mm^3 vs 158,600±51,500/mm^3, p=0.503); between melatonin only group and group with combination therapy (149,900 ± 61,600/mm^3 vs 162,500 ± 51,100/mm^3, p=0.457); and between group with Polymyxin B treatment only and group with combination therapy (158,600±51,500/mm^3 vs 162,500 ± 51,100/mm^3, p=0.364) (table 2, figure 2).

There were significant decreases in platelet count at control group in T2 in comparison to T0 (125,600± 51,200/mm^3 vs 193,800±51,600/mm^3, p=0.022); and in T2 in comparison to T1 (125,600 ± 51,200/mm^3 vs 148,100 ± 62,800/mm^3, p=0.034); as well as in T1 in comparison to T0 (148,100 ± 62,800/mm^3 vs 193,800/mm^3 ± 51,600/mm^3, p=0.039) with paired t-test. Whereas in the group with melatonin only, there were no significant decreases in platelet count after T1 in comparison to T0 (167,500± 71,500/mm^3 vs 199,700±92,500/mm^3, p=0.068) and after T2 in comparison to T1 (149,900 ± 61,600/mm^3 vs 167,500 ±71,500/mm^3, p=0.063). But, there was significant decrease in platelet count after T2 in comparison to T0 (149,900±61,600/mm^3 vs 199,700 ± 92,500/mm^3, p=0.046), (table 2, figure 2). These showed that the platelet count decreased during the progression of endotoxicosis both in control group and in melatonin only group, however it seemed that the decreased platelet was more intense in the control group.

There were no significant decreases in platelet count in the group with Polymyxin B only between T2 and T0 (158,600±51,500/mm^3 vs 195,200±91,300/mm^3, p=0.052), between T2 and T1 (158,600±51,500/mm^3 vs 175,900±72,300/mm^3, p=0.071), and between T1 and T0 (175,900± 72,300/mm^3 vs 195,200 ± 91,300/mm^3, p=0.087) with paired t-test (table 2, figure 2).

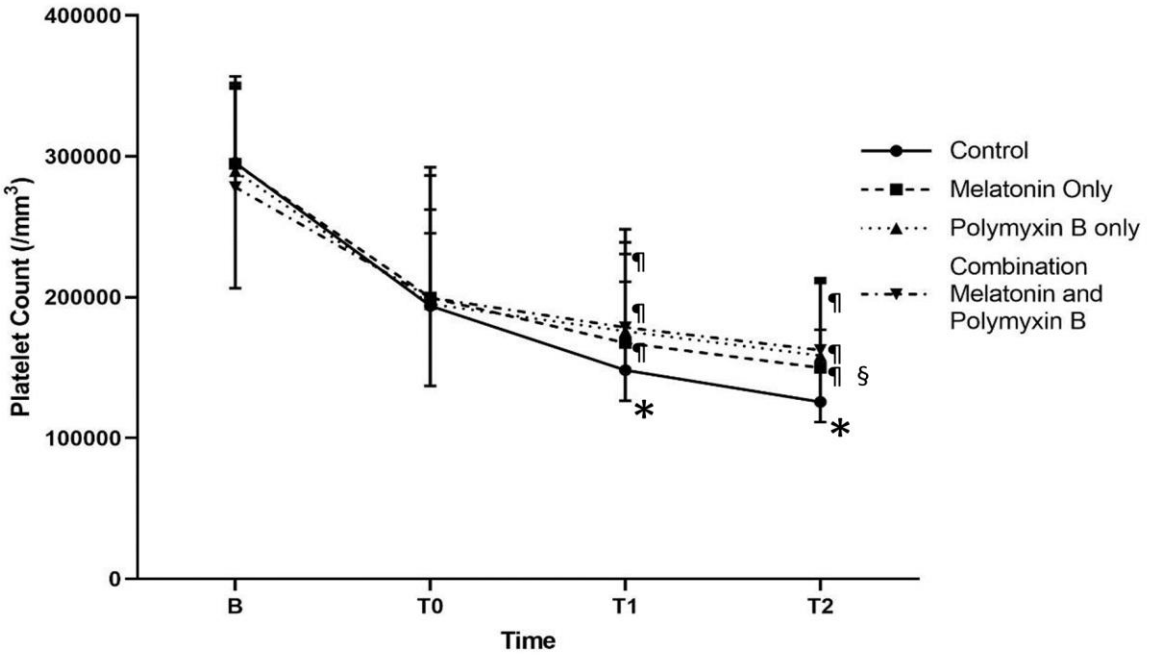
There was no significant decreases in platelet count in the group with combination therapy between T2 and T0 (162,500±51,100/mm^3 vs 199,50±62,600/mm^3, p=0.162), between T2 and T1 (162,500±51,100/mm^3 vs 178,500±52,100/mm^3, p=0.365), and between T1 and T0 (178,500± 52,100/mm^3 vs 199,500 ± 62,600/mm^3, p=0.267) with paired t-test (table 2, figure 2). These showed that the platelet count decreased during the progression of endotoxicosis in control group, while the platelet count were not decreased in group with Polymyxin B only and in group with combination therapy. Polymyxin B and melatonin might prevent the decrease of platelet count after LPS injection.

Figure 1. Leukocyte Count in control group, melatonin only group, polymyxin B only group, and combination group at baseline (B), six hours after LPS injection (T0), one hour after treatment (T1) and two hours after treatment (T2).



*p<0.05 between T2 or T1 in comparison to T0 in control group using paired *t*-test..
¶ p<0.05 between control to melatonin only group, between control to polymyxin B only group, and between control to combination group in T1 and T2 using independent *t*-test.
§ p<0.05 between melatonin only group and combination therapy group in T2 using independent *t*-test.

Figure 2: Platelet Count in control group, melatonin only group, polymyxin B only group, and combination group in baseline (B), six hours after LPS injection (T0), one hour after treatment (T1) and two hours after treatment (T2).



* p<0.05 between T1 or T2 in comparison with T0 in control group using Paired *t*-test.
p<0.05 beteen control to melatonin only group, between control to polymyxin B only group, and between control to combination group in T1 and T2 using independent *t*-test.
§ p<0.05 between T2 and T0 in melatonin only group using paired *t*-test.

Table 1. Leukocyte Count in Control Group, Melatonin Only Group, Polymyxin B Only Group, and Combination Group

Leukocyte Count(/mm^3)	Control Group (C) (n=6)	Melatonin Only Group (M) (n=6)	*p* (between C and M)	Polymyxin B Only Group (P) (n=6)	Combination Group (M+P) (n=6)	*p* (between P and M+P)	*p* (between M and M+P)
Baseline (B)	8,500 ± 1,700; 8,500 (6,800 – 10,300)	8,400 ± 2,100; 8,500 (6,200 – 10,500)	0.724[b]	8,650 ± 1,800; 8,600 (6,800 – 10,550)	8,550 ± 1,900; 8,500 (6,800 – 10,450)	0.657[b]	0.753[b]
Six hours after LPS injection (T0)	10,250 ± 1,900; 10,200 (9,100 – 13,100)	10,150 ± 1,700; 10,100 (8,100 – 11,800)	0.459[b]	10,000 ± 1,300; 10,000 (8,200 – 11,300)	10,100 ± 1,900; 9,900 (7,900 – 12,100)	0.754[b]	0.477[b]
One hour after melatonin administration (T1)	13,900 ± 1,300; 13,500 (12,600 – 15,200)	11,400 ± 3,200; 11,400 (7,200 – 13,600)	0.041*[b]	10,300 ± 1,200; 10,300 (8,800 – 11,500)	9,900 ± 1,200; 9,900 (8,300 – 11,100)	0.643[b]	0.465[b]
Two hours after melatonin administration(T2)	15,700 ± 1,500; 15,500 (14,100 – 17,200)	12,500 ± 2,700; 12,500 (7,600 – 13,100)	0.034*[b]	10,400 ± 1,500; 10,500 (8,500 – 12,900)	9,850 ± 1,100; 9,700 (8,400 – 10,950)	0.563[b]	0.043*b
p (T0 - T1)	0.037*[a]	0.459[a]	-	0.456[a]	0.453[a]	-	
p (T1 - T2)	0.041*[a]	0.434[a]	-	0.437[a]	0.458[a]	-	
p (T0 - T2)	0.017*[a]	0.418[a]	-	0.456[a]	0.455[a]	-	

Value was in mean ± SD; median (min-max), *Significant : $p < 0,05$, [a] *Paired t-Test,* [b] *Independent t-Test, Mann-Whitney*

Table 2. Platelet Count in Control Group, Melatonin Only Group, Polymyxin B Only Group, and Combination Group

Platelet Count (/mm^3)	Control (C) (n=6)	Melatonin Only (M) (n=6)	*p* (C vs M)	Polymyxin B Only (P) (n=6)	Combination (M+P) (n=6)	*p* (P vs M+P)	*p* (M vs M+P)
Baseline (B)	295,500 ± 52,700; 295,500 (242,800 – 348,200)	294,700 ± 62,100; 94,700 (232,600 – 356,800)	0.485[b]	289,500 ± 62,500; 289,500 (227,000 – 352,000)	278,100 ± 71,700; 278,000 (206,300 – 349,800)	0.438[b]	0.435[b]
Six hours after LPS injection (T0)	193,800 ± 51,600; 193,800 (142,200 – 245,400)	199,700 ± 92,500; 199,700 (107,200 – 292,200)	0.278[b]	195,200 ± 91,300; 195,000 (103,000 – 286,500)	199,500 ± 62,600; 199,500 (136,900 – 262,100)	0.276[b]	0.378[b]
One hour after melatonin administration (T1)	148,100 ± 62,800; 148,100 (85,300 – 210,900)	167,500 ± 71,500; 167,500 (96,000 – 239,800)	0.049*[b]	175,900 ± 72,300; 175,000 (103,500 – 248,200)	178,500 ± 52,100; 178,500 (126,400 – 230,600)	0.428[b]	0.348[b]
Two hours after melatonin administration(T2)	125,600 ± 51,200; 125,600 (74,400 – 176,800)	149,900 ± 61,600; 149,900 (88,300 – 211,500)	0.047*[b]	158,600 ± 51,500; 158,500 (107,100 – 210,100)	162,500 ± 51,100; 162,500 (111,400 – 213,600)	0.364[b]	0.457[b]
p (T0 - T1)	0.039*[a]	0.068[a]	-	0.087[a]	0.267[a]	-	
p (T1 - T2)	0.034*[a]	0.063[a]	-	0.071[a]	0.365[a]	-	
p (T0 - T2)	0.022*[a]	0.046*[a]	-	0.052[a]	0.162[a]	-	

Value was in mean ± SD; median (min-max), *Significant : $p < 0,05$, [a] *Paired t-Test,* [b] *Independent t-Test,* [c] *Mann-Whitney*

DISCUSSION

The negative effects of sepsis or endotoxicosis on leukocyte and platelet count have been documented[17,29]. In an acute or emergency care setting, some parameters such as leukocyte count, neutrophil count, lymphocytopenia, neutrophil-lymphocyte count ratio (NLR), and CRP level were predictors of bacteremia[17]. A study revealed that platelets, fibrinogen, and activated partial thromboplastin time (aPTT) at the peak level of CRP to be predictors for survival. An increase in platelets and fibrinogen is linked to survival, whereas an aPTT prolongation is associated with higher mortality[18] However, the mechanisms by which these played role were partially understood.

Our study successfully created endotoxicosis model in wistar rats that was common in sepsis by using LPS injection intraperitoneally. Our study showed that LPS injection had increased leukocyte count and decreased platelet count in six hours after LPS injection (T0) in comparison to baseline (B) in all groups. Our study also successfully showed that there was an increase of leukocyte count and a decrease of platelet count by LPS injection during the progression of endotoxicosis in control group in our endotoxicosis model.

The introduction of bacterial endotoxins, such as LPS, might directly induce immune response and hematologic changes. The increase of leukocyte might be the early body defence mechanism against infection or foreign attacks.[30] This endotoxin LPS was known as a robust bacterial toxin kept within the bacterial cell and released from the cell surface after destruction of the bacterial cell wall.[31] However subsequent study showed that release of LPS from gram negative bacteria did not require the disintegration of the bacterial cell wall, but, LPS was released as a physiological activity of membrane vesicle trafficking in the form of bacterial outer membrane vesicles, which might also contain other virulence factors and proteins[9,31].

Infections and inflammation are fought in the body by both cellular defenses, including leukocyte subtypes: monocytes, macrophages, and neutrophils, and humoral defenses incorporating antibodies and the complement pathways. Recognition of pathogens by extracellular CD14 and toll-like receptors 2 and 4 (TLR-2 and -4) on the

membranes of monocytes and macrophages triggers the release of cytokines to activate cellular defenses.[12] Previously, early leukocytosis was thought to be a protective adaption of the body to current threat, which might increase monocyte mobilisation into the infected-cells, thus improving chances for survival. Recently, prolonged leukocytosis may predict the poor outcome.[12] While low platelet count or thrombocytopenia is a common and multifactorial phenomenon occurring during sepsis. The main causes are decreased platelet production, increased platelets consumption, increased sequestration of platelets in microvessels, increased immune-mediated destruction of platelets, and hemodilution. There are often combination of a decrease in the production, an increase of platelets consumption, and destruction.[29]

The inflammatory cascade in sepsis is complex. A class of pattern recognition molecules on immune cells, such Toll-like receptors (TLRs), responds to the presence of microbiological products as part of innate immunity.[15] TLRs shows a wide variety of functions, but in sepsis, a main role of TLRs engagement is the induction of proinflammatory mediators and activation of nuclear factor (NF-κB).[15] NF-κB is integrally involved in a cascade B formerly known as “cytokine storm” associated with increased expression of proinflammatory cytokines, such as IL-1 and TNF. Other receptors, including those for complements, coagulation factors, and leukotrienes, augment and modify the Toll-like receptor-associated response.[8]

Our study showed that there were no steep increase of leukocyte count and no steep decrease of platelet count in group with melatonin alone, polymyxin B alone, or with combination therapy in comparison to control group at one hour and two hours after treatment. These might imply that melatonin in a certain dose was able to inhibit the increase of leukocyte count and the decrease of platelet count at least in the short-term. However, the mechanisms in how melatonin administration prevented the increase of leukocyte count and decrease of platelet count have not been clearly explained in our study yet.

Our findings in these melatonin properties could be orchestrated by an anti-inflammation and immunomodulation characteristics that improved immune response and inflammatory cytokines[24,32]. Melatonin could modulate nitric oxide (NO) synthesis, iNOS enzyme, COX-2 inhibition, NF-κB activation and the inhibition of neutrophyl infiltration in inflammation. These have been reported to be effective in process against several bacterial and viral infection[20,33]. It also plays role as an antioxidant to improve oxidative stress[25,26], cytoprotective process,[34] anti-apoptosis,[35] anti-corticoid function, and preventing mitochondrial dysfunction as reported elsewhere[20,24].

Wu CC et al have reported that melatonin could prevent endotoxinemia induced by LPS and endotoxin-induced circulatory failure in rats by modulating the production of superoxide in the aorta.[36] As a powerful antioxidant,[37] melatonin antagonised oxidative stress both in a direct and in an indirect way.[21] It prevented free radical-induced damage and increased the activity of several antioxidant enzymes like glutathione-S transferase, glutathione reductase and catalase.[35] Sewerynek et al reported that melatonin might decrease oxidative stress induced by LPS, as evidenced by decreased hepatic malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA).[38] Therefore melatonin is one of the drugs developed as a sepsis therapy[20]. This was consistent with a study by Pierpaoli and Maestroni that reported melatonin's function as an anti-stress hormone[39].

Melatonin showed anti-inflammatory effects by inhibiting the expression and release of COX-2, inducible nitrit oxide synthetase (iNOS), NF-kB, TNF-α, and neutrophil infiltration[34,36], and enhancement in IL-2 production[40]. Melatonin showed immunomodulatory properties and modulatory influence on the NOS and cytokine production in inflammatory and oncostatic processes[41].

Crespo E et al showed that intraperitoneal melatonin administration could inhibit expression of iNOS mRNA in liver and lung[42]. Melatonin decreased lipid peroxidation and counteracted the LPS-induced NO levels in liver and lung. Melatonin prevented LPS-induced endotoxemia and LPS-induced multiple organ dysfunction syndrome in rats[42].

Escames G et al showed that melatonin administration could counteract lipopolysaccharide-induced expression of mitochondrial nitric oxide synthase (mtNOS) and NO production in rats[26], thereby preventing toxicity due to LPS. Melatonin could prevent the mitochondrial failure that occurs during endotoxemia[26].

Our study showed that group with polymyxin B could prevent leukocytosis and thrombocytopenia. We hypothesized that the better leukocyte and platelet profiles might be correlated with its direct anti-endotoxins and improved cytokine secretion. Polymyxin B might be an attractive peptide in combating endotoxic shock through mediating the neutralization of LPS. Harm et al reported an in vitro inactivation of endotoxins by polymyxin B (PMB).[23] The in vitro LPS–PMB complex had lower inflammatory activity in freshly drawn blood samples, which resulted in reduced cytokine secretion.[23] Inactivation of endotoxins by intravenous polymyxin B infusion might be applied to overcome the urgent need for endotoxin elimination in treatment of sepsis or endotoxicosis[23].

However, we still not certain whether therapy with melatonin alone might be sufficient to inhibit leukocytosis and thrombocytopenia in the longer term during further sepsis or endotoxicosis progression, in which several complications might usually appear. Furthermore, we still can not elucidate whether we will need a higher dose of melatonin to inhibit the increase of leukocyte count and the decrease of platelet count in the longer term.

It is challenging to examine the effect of melatonin administration as a prevention or prophylaxis therapy in endotoxicosis or sepsis. We also need further study in the effects of melatonin with multilevel doses, with the varying duration of exposure, and with larger study subjects.

CONCLUSION

An adjuvant therapy with melatonin could prevent the elevation of leukocyte count and the decrease of platelet count in Wistar rats endotoxicosis model. Therefore, if confirmed by further research, melatonin might have a role in management of endotoxicosis.

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