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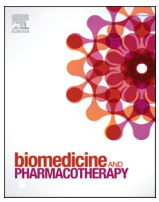

Methylene blue as a potential intervention in sepsis: Effects on survival and microcirculation in rat models of sepsis

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ABSTRACT

Sepsis is a life-threatening condition characterized by systemic inflammation and microcirculatory dysfunction. Methylene blue (MB), a compound with known antioxidant and anti-inflammatory properties, has been proposed as a potential therapeutic agent. This study aimed to investigate the effects of MB on survival rates and the preservation of mesenteric microcirculation in a rat model of endotoxemia. A total of 36 rats underwent cecal ligation and puncture (CLP) surgery to induce varying degrees of sepsis: mild (4 perforations), moderate (10 perforations), and severe (20 perforations). Animals received intravenous treatment with either MB (4 mg/kg) or saline. Survival was monitored for ten days. Additionally, intravital microscopy was used to assess leukocyte rolling and adhesion in mesenteric vessels following lipopolysaccharide (LPS)-induced sepsis. The experimental groups included saline, LPS + saline, MB + saline, LPS + MB, and MB + LPS. MB treatment significantly improved survival in the severe sepsis group, with a 30 % survival rate at ten days ($p = 0.02$, 95 % CI: 0.12–0.48), whereas all animals in the severe sepsis + saline group died within nine days. No significant survival benefit was observed in the mild and moderate sepsis groups (mild sepsis: $p = 0.45$, 95 % CI: 0.08–0.34; moderate sepsis: $p = 0.32$, 95 % CI: 0.15–0.51). In the LPS-induced model, treatment with both LPS and MB significantly reduced leukocyte rolling and adhesion ($p < 0.001$, 95 % CI: 0.45–0.75 for rolling; $p < 0.03$, 95 % CI: 0.30–0.60 for adhesion), with values comparable to those of the control group. In contrast, MB alone had no effect on leukocyte rolling or adhesion.In summary, MB significantly improved survival in severe sepsis and inhibited leukocyte migration in mesenteric vessels. These findings suggest that MB may protect the microcirculation and enhance survival under severe septic conditions, representing a promising therapeutic approach for sepsis management.

1. Introduction

Sepsis is a global health challenge and remains the leading cause of mortality worldwide. It occurs when the body's response to an infection triggers a widespread inflammatory cascade that can cause severe damage to multiple organs and systems [1]. If left untreated, sepsis can lead to life-threatening complications and death, with mortality rates significantly increasing as the condition progresses [2]. The hallmark of sepsis is an exaggerated and uncontrolled inflammatory response, which results in endothelial dysfunction, increased vascular permeability, and generalized vasodilation, often culminating in septic shock [3]. Septic shock is characterized by hypoxia, multiple organ failure, and dysregulated immune responses, and is driven by complex inflammatory pathways, including the release of proinflammatory cytokines, activation of immune and complement cells, and excessive nitric oxide (NO) production [4].

Sepsis-induced cardiomyopathy (SIC) is a major complication of sepsis, often manifesting as reversible myocardial dysfunction. This condition is characterized by impaired cardiac contractility despite normal or increased cardiac output and is associated with increased

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mortality [5]. The pathophysiology of SIC involves a complex interplay of inflammatory mediators, mitochondrial dysfunction, and calcium dysregulation, in which proinflammatory cytokines such as TNF-α and IL-1β play a crucial role in suppressing myocardial contractility [6]. The ability of MB to reduce inflammatory mediators and oxidative stress may offer a novel strategy for protecting cardiac function during sepsis. Intravital microscopy evidence also suggests that MB may act as a vasopressor-sparing agent in sepsis-induced vasoplegia, further supporting its potential as a therapeutic intervention in this setting [7].

Current management of sepsis involves standardized treatment protocols, including intravenous fluid resuscitation, early antibiotic administration, and the use of vasopressors. In cases of vasopressor-resistant shock, low-dose corticosteroids are occasionally used [8]. However, despite these therapeutic strategies, the prognosis for severe sepsis and septic shock remains poor, with high mortality rates still observed [9,10]. This has led to ongoing research aimed at identifying new or repurposed drugs to improve patient outcomes.

One such candidate is MB, a compound traditionally used to treat methemoglobinemia, carbon monoxide poisoning, and other conditions. MB has gained attention for its potential to improve outcomes in sepsis, particularly in cases that are resistant to conventional therapies [11]. Its pharmacological action is thought to involve the inhibition of guanylate cyclase and nitric oxide synthase, thereby reducing NO production and subsequently improves hemodynamics [12]. Additionally, MB possesses antioxidant and anti-inflammatory properties, which can mitigate inflammation and oxidative damage commonly associated with sepsis [13]. Recent studies have also explored its efficacy when used in combination with standard therapies, showing promising results in improving clinical outcomes in septic shock [14].

Despite promising results from preliminary studies, the specific effects of MB on survival rates and microcirculation in sepsis have not been fully elucidated. In particular, the mechanisms by which MB modulates leukocyte migration and endothelial function in sepsis are poorly understood. Furthermore, while MB has shown potential in reducing mortality in experimental models, its efficacy across different stages of sepsis (e.g., mild, moderate, and severe) and its long-term effects on organ recovery and microcirculatory preservation, remain to be fully investigated. This study aims to address these gaps by evaluating the effects of MB on survival and microcirculation in rat models of sepsis.

Animal models, such as the cecal ligation and puncture (CLP) model and the lipopolysaccharide (LPS)-induced endotoxemia model, are commonly used to study the mechanisms of sepsis and its complications. The CLP model, which mimics polymicrobial sepsis, is particularly useful for investigating survival outcomes and the efficacy of therapeutic interventions. The LPS model, by contrast, is valuable for studying the inflammatory cascade and leukocyte-endothelium interactions, which are key aspects of sepsis pathology [15].

Recent research suggests that MB could have a significant impact on the inflammatory response in sepsis by modulating cellular functions, particularly those of leukocytes. Leukocyte migration is a critical component of both innate and adaptive immune responses, facilitating immune surveillance and the body's defense against infection [16]. By altering leukocyte signaling pathways involved in migration and survival, MB may offer a means of mitigating the excessive leukocyte activity observed in sepsis, which contributes to tissue damage and organ dysfunction.

In light of these considerations, our study aimed to investigate whether MB treatment could improve survival rates in a rat model of sepsis induced by CLP and preserve mesenteric microcirculation by reducing leukocyte migratory activity in an LPS-induced endotoxemia model. We hypothesized that MB treatment will improve survival rates and preserve mesenteric microcirculation in rats with sepsis, offering a promising therapeutic approach for this life-threatening condition. This research explores the potential of MB as a therapeutic agent in sepsis, offering insight into its effects on both survival outcomes and immune function.

2. Methods

2.1. Animals

Adult male Hannover rats (8–12 weeks old, 150–220 g) were obtained from the Animal Facility of the University of São Paulo, Ribeirão Preto Campus (USP). The animals were housed in a controlled environment with a temperature of 22–25°C, a 12-h light/dark cycle, and had ad libitum access to food and water. All experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Ribeirão Preto Medical School (CEUA n.°1274/2023). Only healthy animals with no prior history of infection or inflammatory conditions and with normal physiological parameters before the experimental procedures were included in the study. Rats that did not recover from anesthesia, dispalyed signs of extreme distress (e.g., severe weight loss > 20 %, respiratory distress), showed clinical signs of infection prior to the experiment, or presented surgical complications such as excessive bleeding or unintended intestinal perforations were excluded from the study.

2.2. Sepsis induction by cecal ligation and puncture (CLP)

Sepsis was induced using the cecal ligation and puncture (CLP) model, as previously described [17,18]. Rats were randomly assigned to receive either MB(n = 20) or saline (n = 18). The MB dose (4 mg/kg, intravenous bolus) was determined based on previous studies [19]. For the procedure, rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (90/10 mg/kg). Under sterile conditions, a midline laparotomy (2 cm) was performed to expose the cecum and mesentery. Before sepsis induction, animals received a pretreatment injection via the vena cava with either methylene blue (4 mg/kg, 20 μL) or saline (20 μL). Sepsis was then induced by cecal ligation and puncture (CLP): the cecum was ligated below the ileocecal junction, ensuring no intestinal obstruction, and perforations were made with a 16-gauge needle to modulate severity—four perforations for mild sepsis (LSS), ten for moderate sepsis (MSS), and 20 for severe sepsis (SSS), based on previous literature [20]. The cecum was repositioned, and the abdominal wall was immediately closed with sutures. Postoperatively, all animals received 5 mL of subcutaneous saline for fluid resuscitation.

Animals were monitored for clinical signs of sepsis, including lethargy, piloerection, tachypnea, and tachycardia. The following exclusion criteria were applied: (1) failure to regain consciousness after anesthesia, (2) severe distress requiring humane euthanasia, (3) surgical complications (e.g., excessive bleeding, unintentional intestinal perforations), or (4) death prior to data collection. The classification of sepsis severity was based on previously reported survival rates.

2.3. Intravital microscopy

Intravital microscopy was used to assess microcirculatory integrity by quantifying leukocyte rolling and adhesion in the mesenteric microvasculature of rats subjected to different experimental treatments. The experimental groups were: Saline (rats receiving intravenous saline only), LPS + Saline (rats receiving LPS followed by saline administered 10 min later), LPS + MB (rats receiving LPS followed by MB 10 min later), MB + LPS (rats pre-treated with MB 10 min before LPS), and MB (rats receiving MB alone). Procedures followed previously established protocols with modifications [21,22]. Animals were anesthetized via intraperitoneal injection of urethane (0.4 g/mL), and a catheter was inserted into the femoral vein for precise intravenous administration of substances. Systemic inflammation was induced by LPS (10 mg/kg, IV), and methylene blue (4 mg/kg, IV) or saline was administered according to the experimental design. A lateral incision in the abdominal skin was made to open the peritoneal cavity and expose the mesenteric

microcirculation. Animals were placed on a thermoregulated platform maintained at 37°C, and the exposed tissue was continuously irrigated with warm sterile saline to preserve tissue viability. The mesentery was carefully externalized and positioned on a transparent pedestal to allow direct visualization.

Microcirculatory assessment was performed using a brightfield trinocular microscope (Nikon Eclipse Ti-E, Japan) equipped with a high-resolution digital camera (Hamamatsu ORCA-Flash 4.0, Japan). Real-time video recordings of the mesenteric microcirculation acquired at 30 frames per second for 10 min using NIS-Elements AR software (Nikon, Japan). Post-capillary venules with diameters between 10 and 18 μm were selected for analysis. Leukocyte dynamics were quantified using ImageJ software (National Institutes of Health, USA) with the TrackMate plugin for cell tracking. Rolling leukocytes were defined as cells moving along the endothelium at a velocity slower than that of the blood flow and were expressed as the number of rolling cells per 10 μm of vessel length per minute. Adherent leukocytes were defined as cells that remained stationary for more than 30 s and were expressed as the number of cells per 100 μm^2 of endothelial surface area. Three independent observers who were blinded to the experimental groups to ensure consistency and minimize bias performed video analyses.

2.4. Statistical analysis

Data are presented as means ± SEM. Comparisons between three or more groups were performed using a one-way ANOVA followed by Tukey's post hoc test. Survival analysis was conducted using the log-rank (chi-square) test. A p-value of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software Corporation, La Jolla, California, USA).

3. Results

3.1. MB improved survival in severe sepsis

MB administration significantly improved survival outcomes in the severe CLP sepsis model (Fig. 1). Kaplan-Meier analysis revealed a 30 % survival rate at 10 days post-intervention in MB-treated animals (dashed blue line; $p = 0.02$ versus controls). In sharp contrast, the saline-treated control group (solid black line) experienced 100 % mortality by day 9. This survival benefit, achieved despite the model's extreme lethality, suggests that MB may be particularly valuable in cases of refractory septic shock, demonstrating its therapeutic potential in this challenging clinical scenario.

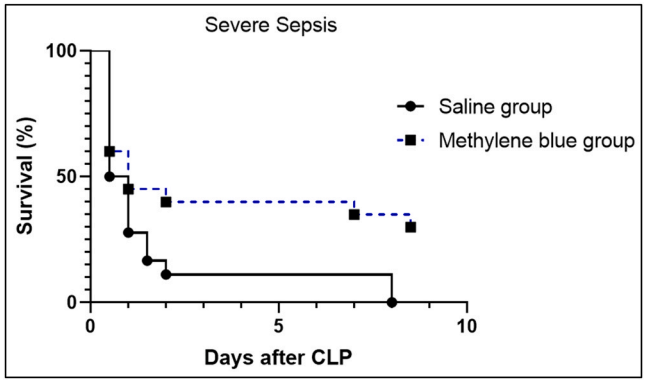

Fig. 1. Survival curves for rats that underwent severe CLP sepsis (20 punctures of the cecum). The solid line represents the group treated with saline solution (Saline Group), while the dashed line represents those treated with methylene blue (MB Group). The treatment was administered immediately before the CLP model was induced. The log-rank test showed a statistically significant difference between the two groups ($n = 18$ per group, $p = 0.02$).

In mild sepsis models (Fig. 2), MB treatment showed no statistically significant survival benefit compared to saline controls ($p > 0.05$). While the MB group exhibited a 21 % survival rate at 10 days versus 0 % in saline-treated animals, this difference did not reach statistical significance. The complete mortality observed in control animals by day 10 confirms the model's validity while underscoring MB's apparent limited efficacy in mild sepsis presentations

In the moderate sepsis model (Fig. 3), MB treatment failed to demonstrate a statistically significant survival benefit compared to saline controls ($p > 0.05$). Although the MB group showed a numerically higher survival rate (43 % vs 9 % in controls at 10 days), this apparent difference did not reach statistical significance. The substantial mortality in both groups (57 % MB vs 91 % saline) confirms the model's pathogenicity while highlighting the need for more potent interventions in moderate sepsis cases.

3.2. MB downregulated endothelial leukocyte rolling and adhesion

Rats administered LPS exhibited a notable increase in leukocyte rolling (Fig. 4) and adhesion (Fig. 5) within mesenteric vessels, contrasting with the saline group that showed physiological rolling ($p = 0.001$) and adhesion ($p = 0.01$).

The rats that received only MB (MB + saline) demonstrated similar parameters for rolling (Fig. 4) as those that received saline. The group that received LPS plus saline intervention (LPS + SAL) exhibited a significantly higher value of leukocyte rolling compared to the saline group (SAL) ($p = 0.0001$, Fig. 4). However, when rats were treated with MB and exposed to LPS (MB + LPS, Fig. 4), the rolling of leukocytes in the mesentery decreased significantly compared to the LPS group ($p < 0.0001$, Fig. 4). The groups that received LPS with MB intervention (LPS + MB) demonstrated lower leukocyte rolling than the LPS group ($p = 0.0001$, Fig. 4). The groups that received MB before LPS (MB+LPS) exhibited decreased leukocyte rolling compared to the LPS group ($p = 0.001$, Fig. 4). MB treatment induced similar downregulation in leukocyte rolling (Fig. 4) when administered before or after sepsis induction through LPS, indicating that treatment with MB significantly reduces rolling leukocytes in the mesentery.

The groups MB + saline showed similar parameters for leukocyte adhesion (Fig. 5) as those that received saline. The group that received LPS plus saline intervention (LPS + SAL) exhibited a significantly higher level of leukocyte adhesion compared to the saline group (SAL) ($p = 0.01$, Fig. 5). The group that received MB (MB + saline) showed leukocyte adhesion similar to the saline group (Fig. 5). However, the adhesion of leukocytes in the mesentery decreased significantly in the groups where rats were exposed to LPS and treated with MB (MB + LPS) compared to the LPS+saline group ($p < 0.03$, Fig. 5). MB treatment induced downregulation in leukocyte adhesion (Fig. 5) after sepsis induction through LPS. However, when MB was administered before sepsis induced by LPS, there was no significant difference compared to the LPS group (Fig. 5).

4. Discussion

This study investigated the therapeutic potential of MB in experimental models of sepsis, with a focus on its effects on survival and microcirculatory integrity. Our results demonstrate that MB significantly improves survival in severe sepsis induced by CLP and effectively attenuates leukocyte-endothelium interactions in lipopolysaccharide (LPS)-induced endotoxemia. These findings support the potential of MB as a therapeutic adjunct in sepsis, particularly during advanced stages characterized by pronounced systemic inflammation and endothelial dysfunction.

The 30 % survival rate observed in the severe CLP model following MB treatment is consistent with previous studies showing that MB can reduce mortality in experimental sepsis, especially when the

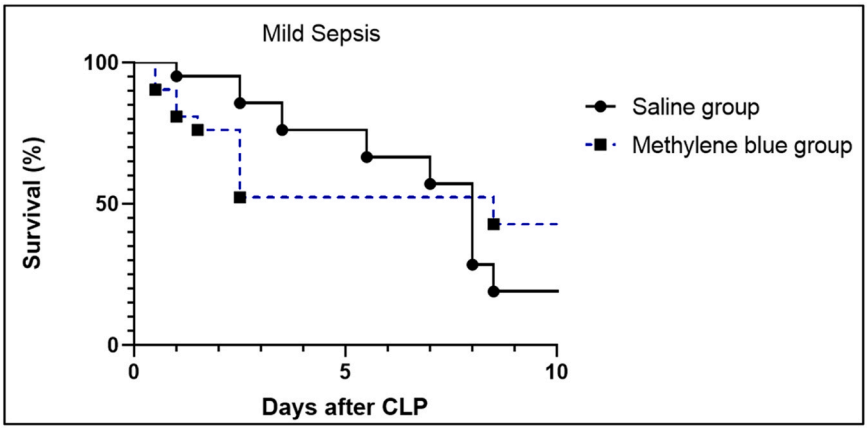

Fig. 2. The graph displays survival curves for rats that underwent mild CLP sepsis (four cecum punctures). The solid line represents the control group treated with saline solution, while the dashed line represents the group treated with MB. The treatment was administered minutes before inducing the CLP model. The study included 21 rats per group, and the log-rank test was used to determine statistical significance.

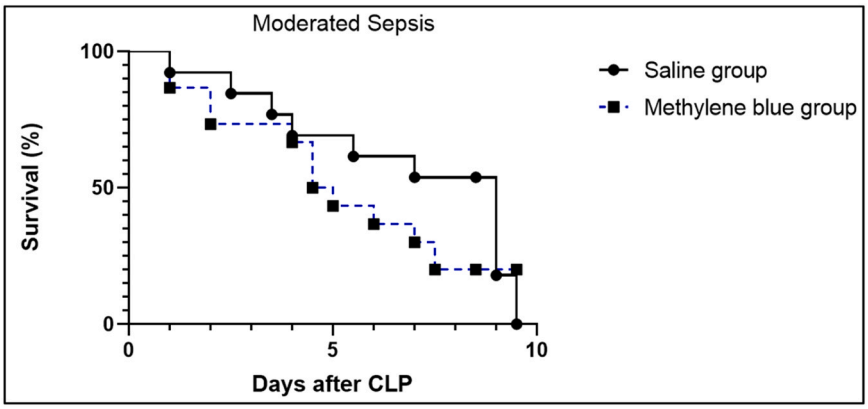

Fig. 3. The graph displays survival curves for rats that underwent moderate CLP sepsis (10 punctures of the cecum). The solid line represents the control group treated with saline solution, while the dashed line represents the group treated with MB. The treatment was administered minutes before the CLP model was induced. The log-rank test was used to determine statistical significance (n = 25 per group).

inflammatory burden is high [23,24]. MB's efficacy is largely attributed to its pharmacological inhibition of nitric oxide synthase and soluble guanylate cyclase, leading to reduced NO production. This leads to improved vascular tone, stabilization of hemodynamics, and attenuation of oxidative stress—key pathological mechanisms in septic shock [12,13,25]. These findings reinforce MB's potential as a targeted therapy for septic shock, where excessive NO-mediated vasodilation and oxidative damage are central to organ failure.

In the LPS model, MB significantly reduced leukocyte rolling and adhesion in the mesenteric microcirculation. These findings suggest that MB not only restores vascular function but also modulates the inflammatory response at the cellular level, likely through the downregulation of adhesion molecules such as ICAM-1 and VCAM-1 [26,27]. These effects are particularly relevant in sepsis, where endothelial activation and excessive leukocyte infiltration drive tissue damage and multi-organ dysfunction [18,28]. Interestingly, MB failed to significantly improve survival outcomes in mild-to-moderate sepsis models. This finding supports the hypothesis that MB's therapeutic benefits become clinically significant only in settings of severe inflammatory dysregulation. Further research should establish (1) the precise inflammatory threshold required for MB efficacy, (2) optimal therapeutic windows, and (3) dose-response relationships [1].

Recent research on sepsis has increasingly emphasized the correlation between leukocyte dynamics and survival outcomes, offering a more integrated understanding of prognosis. Studies show that early changes in white blood cell, neutrophil, and lymphocyte counts are closely linked to survival. Notably, rising lymphocyte counts and falling neutrophil-to-lymphocyte ratios within the first three days of ICU admission correlate with improved 28-day survival in septic shock patients [29]. This immune trajectory reflects the restoration of adaptive immunity and attenuation of hyperinflammation, both of which are critical determinants of outcome.

These findings assessment align with growing evidence that cytokine profiles, immune cell function, and leukocyte-endothelial interactions are reliable prognostic indicators in sepsis. Meta-analyses report that survivors exhibit significant reductions in pro-inflammatory cytokines (e.g., IL-6 and TNF-α) after treatment, whereas non-survivors do not [30]. Similarly, elevated activation markers on neutrophils and monocytes—and their interaction with endothelial cells—are associated with worse outcomes, likely reflecting an excessive compensatory anti-inflammatory response [31].

In addition, low circulating levels of total cholesterol, HDL-C, and LDL-C have been strongly associated with increased mortality in sepsis and critical illness, suggesting that lipid metabolism may also serve as a valuable prognostic marker [32]. These findings are further supported by machine learning studies identifying gene-expression signatures predictive of persistent organ dysfunction in sepsis [33], impaired CD8 + T cell activation driven by SIGLEC5 [34], and the prognostic value of hemodynamic indices like the blood pressure response index in septic shock [35].

Additional prognostic markers have emerged from metabolic and vascular studies. Low circulating levels of total cholesterol, HDL-C, and

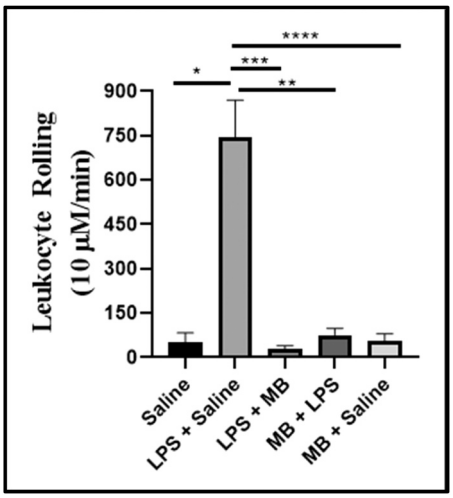

Fig. 4. Leukocytes rolling in the mesenteric microcirculation of rats over ten minutes. The groups included Saline (received intraperitoneal saline before the counting period), LPS + Saline (received intraperitoneal LPS followed by saline treatment), LPS + MB (received intraperitoneal LPS followed by methylene blue treatment), MB + LPS (received pre-treatment with MB followed by intraperitoneal LPS), and MB (received methylene blue only). Saline group demonstrated physiologically normal values, while the LPS group exhibited significantly elevated values. The LPS + MB and MB + LPS groups showed significant reductions in leukocyte counts, indicating a beneficial influence of methylene blue, regardless of timing, on leukocyte rolling. The group that received only MB showed normal values of leukocyte rolling. ($p > 0001$ using one-way ANOVA). Leukocyte rolling data expressed as cell rolling per 10 micrometers per minute (µm/min). Data are presented as mean ± SEM. ANOVA followed by the Tukey post hoc test was used to compare three or more groups. *($p = 0.001$), **($p = 0.001$), ***($p = 0.001$), ****($p = 0.001$).

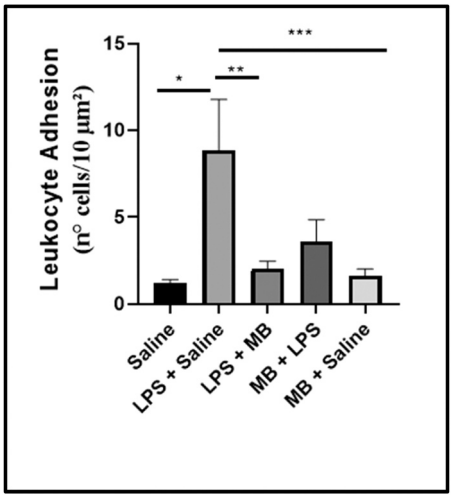

Fig. 5. Leukocyte Adhesion in the mesenteric microcirculation of rats. The groups included Saline (received intraperitoneal saline before the counting period), LPS + Saline (received intraperitoneal LPS followed by saline treatment), LPS + MB (received intraperitoneal LPS followed by MB treatment), MB + LPS (received pre-treatment with methylene blue followed by intraperitoneal LPS), and MB (received methylene blue only). Leukocyte adhesion was analyzed as remaining adhered to the vascular endothelium for more than 30 s and expressed as the number of adhered cells per 10 square micrometers (μm^2). Notably, the Saline group demonstrated physiologically normal values, while the LPS group exhibited significantly elevated values. The group that received only MB showed normal values of leukocytes adhesion ($p > 0001$ using one-way ANOVA), Data are presented as mean ± SEM. ANOVA followed by the Tukey post hoc test was used to compare three or more groups. *($p = 0.01$), ** ($p = 0.02$), ***($p = 0.03$).

LDL-C have been independently associated with increased mortality in sepsis and critical illness [32]. Moreover, immunomodulatory strategies have shown therapeutic promise. For instance, selective enhancement of macrophage antibacterial responses via Keap1-Nrf2 inhibition significantly attenuates sepsis severity and supports localized host defense without exacerbating systemic inflammation [27]. Together, these studies underscore the importance of integrating leukocyte kinetics, cytokine signatures, immune cell function, metabolic status, and vascular responsiveness into a unified framework for risk stratification and personalized therapy in sepsis.

Several limitations should be acknowledged. Although rodent models offer valuable mechanistic insights, they cannot fully replicate the complexity and clinical heterogeneity of human sepsis. The translation of these findings requires validation in large animal models or human clinical studies to establish MB's therapeutic potential. Furthermore, our evaluation of a single MB dose precludes the assessment of optimal therapeutic regimens. Future investigations should examine dose-response relationships, multiple administration protocols, and long-term outcomes, particularly regarding organ protection, functional recovery, and potential post-sepsis complications including cognitive deficits and persistent renal impairment [36,37].

MB's potential as a vasopressor-sparing agent in vasopressor-refractory septic shock significantly bolsters its clinical value [11,38]. Furthermore, combination strategies pairing MB with complementary anti-inflammatory or immunomodulatory therapies may yield synergistic benefits, potentially improving outcomes in critically ill sepsis patients by simultaneously targeting multiple pathological pathways [39,40]. Collectively, our experimental data demonstrate that MB confers significant survival benefits and microcirculatory protection in severe sepsis models. These findings position MB as a promising therapeutic candidate, particularly for cases characterized by profound hemodynamic instability and endothelial dysfunction. Moving forward, critical research priorities should focus on: (1) defining patient-specific response profiles across the sepsis severity spectrum, (2) elucidating time-dependent therapeutic effects through rigorous pharmacokinetic -and- pharmacodynamic studies, and (3) developing optimized combination protocols that simultaneously target inflammatory, coagulopathic, and microvascular components of sepsis pathogenesis.

In summary, our findings provide strong experimental evidence that MB is effective in improving survival and preserving microcirculation in severe sepsis. Further studies are essential to explore its application in broader clinical scenarios, determine the ideal therapeutic window, and validate its use in combination regimens targeting the multifactorial nature of sepsis.

5. Conclusion

In conclusion, the results of this study underscore the therapeutic potential of MB in improving survival rates in severe sepsis models induced by CLP, as well as in reducing leukocyte migration in LPS-induced endotoxemia. While promising, MB demonstrated limited efficacy in mild-to-moderate sepsis models, highlighting the need for further exploration of its therapeutic window and optimal dosing regimens. These findings provide a robust foundation for future research aimed at refining MB's application across various stages of sepsis, particularly regarding administration timing, combination therapies, and the translation of preclinical results to human clinical trials.

CRediT authorship contribution statement

Evora Paulo R.: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luis-Silva Fabio:** Writing – original draft, Visualization, Validation, Investigation. **Becari Christiane:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision,

Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ribeiro Mauricio S.:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dantas Pedro Brüch:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mestriner Fabiola:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dugaich Vinicius Flora:** Methodology. **Michelon-Barbosa Jessyca:** Methodology, Investigation, Data curation.

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Declaration of Competing Interest

The authors declare that there are no conflict of interest

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