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Protective effects of avasopasem manganese (GC4419) against sepsis-induced acute lung injury: A comprehensive experimental study

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Abstract

Background: Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection, characterised by excessive oxidative stress and cytokine release. This study evaluated the protective effects of avasopasem manganese (AVA), a superoxide dismutase mimetic, on sepsis-induced lung injury in a murine model.

Methods: Sepsis was induced in mice via cecal ligation and puncture (CLP). The study included five groups ($n = 8$/group): Sham (control), CLP (Sepsis) and CLP + AVA at doses of 2.5, 5 or 10 mg/kg. Serum and lung tissue samples were analysed for pro-inflammatory cytokines (tumour necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6), oxidative stress markers (malondialdehyde, MDA) and antioxidant enzyme activity (superoxide dismutase, SOD; glutathione, GSH; catalase, CAT). Quantitative real-time PCR (qRT-PCR) assessed cytokine mRNA expression, while histopathological examination evaluated lung tissue damage.

Results: Serum and lung tissue levels of TNF-α, IL-1β, IL-6 and MDA were lowest in the healthy control group and highest in the sepsis group ($p < .001$). A significant dose-dependent decrease in cytokine levels was observed with increasing doses of AVA. Regarding antioxidants, SOD, GSH and CAT enzyme activities were highest in the control group and lowest in the sepsis group ($p < .05$). A significant increase in antioxidant enzyme activity was observed with increasing doses of AVA. In molecular analyses, the expression levels of TNF-α, IL-1β and IL-6 were highest in the sepsis group, while relative messenger RNA (mRNA) expression results changed inversely with the drug dosage. Histopathological analyses revealed inflammation, edema and hyaline membrane formation in the sepsis group, whereas increasing doses of the drug showed improvement in lung tissue.

Conclusions: AVA demonstrated a significant protective effect against sepsis-induced lung injury through its antioxidant and anti-inflammatory properties.

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The most effective dose was determined to be 10 mg/kg. These findings suggest the potential use of AVA as an adjunctive agent in sepsis treatment.

KEYWORDS

acute lung injury, antioxidants, avasopasem manganese, cecal ligation and puncture, cytokines, sepsis

1 | INTRODUCTION

Sepsis is a life-threatening organ dysfunction resulting from the host's dysregulated and excessive immune response to infection. This condition can also be defined as systemic inflammatory response syndrome (SIRS) caused by infection. Sepsis, if not diagnosed and treated early, can result in septic shock, multiple organ failure and death.[1] Despite advances in sepsis treatment and management, it remains a primary cause of hospital deaths.[2,3]

Reactive oxygen species (ROS) and cytokines formed during cellular respiration or various metabolic by-products play a fundamental role in the development of sepsis.[4] Major cytokines such as TNF-α, IL-1β and IL-6 stimulate the inflammatory response and promote ROS formation, which leads to organ damage.[5] ROS exhibit pro-inflammatory activity and cause endothelial damage, leukocyte infiltration, cytokine release, mitochondrial dysfunction, lipid peroxidation and DNA damage. All of these steps result in excessive free radical production and oxidative stress. This condition explains organ damage in sepsis.[6,7] In the body, antioxidant mechanisms develop a defence system against free radicals to reduce free radical toxicity. These include antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase, catalase (CAT), thioredoxin reductase, peroxiredoxins (Prx) and NAD(P)H: ubiquinone oxidoreductase (NQO1).[8] Among these, SOD and CAT are the primary defence line enzymes against free radicals in the cell.[9–11]

Superoxide anion is converted to H_2O_2 (hydrogen peroxide) by SOD, and hydrogen peroxide is inactivated by glutathione peroxidase or catalase. Avasopasem manganese (AVA) (GC4419), which exhibits SOD mimetic activity, has been studied, especially in recent years, and acts through this pathway. AVA exerts antioxidant and anti-inflammatory effects by converting superoxide radicals into hydrogen peroxide. Studies on AVA are current research from the last 5 years, and its therapeutic efficacy is being investigated.[12–15]

Currently, there is no effective treatment for sepsis other than supportive treatments such as fluid replacement, antibiotic therapy and vasopressors. The development of new treatment strategies, especially in animal models, is of great importance.[1] Thus, the potential ameliorative effect of SOD mimetic AVA on sepsis is of great interest.

In this study, we aimed to investigate the potential therapeutic effects of AVA on serum and lung tissue in a mice model of cecal ligation and puncture (CLP)-induced sepsis, using molecular, biochemical and histopathological analyses.

2 | METHODS

2.1 | Animals and Groups

This study was approved and authorised by the Ataturk University Animal Experiments Local Ethics Committee (decision number E-75296309-050.01.04-2200200553, dated 04.07.2022), which operates in line with European (EU) Directive 2010/63/EU. This study was conducted at the Ataturk University Medical Experimental Application and Research Centre. The number of mice to be used in the study was determined as 8 in total as a result of the power analysis (α: .05, power: .85, effect size: .40) using the G*Power 3.1.9.4 programme. Forty 6–8-week-old female BALB-C mice were used in the study. The mice were maintained under controlled temperature conditions of 22 ± 1°C and a relative humidity of 50%–60% in a 12-h light/dark cycle and were fed with tap water and pellet feed.

2.2 | Experimental design

The 40 mice were randomly separated into five groups ($n = 8$ per group):

1. Control: Group receiving only distilled water
2. CLP: Group in which sepsis was induced by the CLP method and did not receive drug treatment
3. CLP + AVA 2.5: Group in which sepsis was induced by the CLP method and received 2.5 mg/kg Avasopasem treatment

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4. CLP + AVA 5: Group in which sepsis was induced by the CLP method and received 5 mg/kg Avasopasem treatment
5. CLP + AVA 10: Group in which sepsis was induced by the CLP method and received 10 mg/kg Avasopasem treatment

2.3 | Administration of avasopasem manganese (GC4419), a SOD mimetic

In this study, the AVA compound, a SOD mimetic that we consider to be a potential target for reducing sepsis damage, was used. AVA was prepared at a stock concentration of 4.83 mg/mL in saline with 10 mmol/L sodium bicarbonate (pH 7.1–7.4). AVA was administered intraperitoneally (I.P.) at doses of 2.5, 5 and 10 mg/kg to the groups mentioned above.[16]

2.4 | Cecal ligation and puncture (CLP)-induced sepsis model

Mice were anaesthetised by intraperitoneal (ip) administration of a combination of ketamine (20 mg/kg)/xylazine (10 mg/kg).[17] The abdominal regions of the mice were shaved, and a 1 cm midline laparotomy was performed under aseptic conditions. The cecum was tightly ligated at the midpoint with a 4/0 silk suture, and then four holes were opened in the cecum away from the ligation point with a 21-gauge needle from the opposite side of the mesentery. Faeces were squeezed out of the cecum. Then, the cecum was placed into the peritoneal cavity, and the abdomen was closed with 3/0 silk suture. All mice were given .5 mL saline for fluid resuscitation during the surgical procedure, and Avasopasem treatment at the doses specified above was administered to the drug-treated groups 1 h after the CLP procedure.[18] After 18 h following the sepsis induction with CLP, all groups were anaesthetised with 100 mg/kg ketamine and 10 mg/kg xylazine, and cardiac blood and lung tissue samples were obtained.[19]

2.5 | Preparation and analyses of samples

Cardiac blood samples taken from the mice were collected into gelled vacuum tubes, and serum samples were obtained after centrifugation at 14 000 revolutions per minute (rpm) for 10 minutes. Half of the lung tissue samples were fixed in formaldehyde solution for histopathological studies. The remaining lung tissues were cooled with liquid nitrogen for molecular studies and ground using a TissueLyser II grinding set device (Qiagen, Hilden, Germany). For enzyme-linked immunosorbent analysis (ELISA) and biochemical measurements, 50 mg was taken from each of the ground tissue samples and homogenised again in 1 mL phosphate buffer (100 Mm, pH 7.0) using tungsten beads in a TissueLyser II grinding set device. The homogenate was centrifuged in an ultracentrifuge at 14 000 rpm for 5 min, and the resulting homogenate was used in ELISA measurements and biochemical tests.

2.6 | ELISA analyses of lung tissue and serum samples

TNF-α, IL-1β and IL-6 levels in the supernatant obtained from lung tissue and serum samples obtained from blood were measured by the ELISA method according to the kit protocol; results were obtained by spectrophotometric measurement.[20]

2.7 | Biochemical analyses of lung tissue and serum samples

SOD activity, glutathione (GSH), CAT activity and malondialdehyde (MDA) levels in the supernatant obtained from lung tissue and serum samples obtained from blood tissue were measured as described in the biochemical procedures, and results were obtained by spectrophotometric measurement.

2.8 | PCR analysis

In our study, TNF-α, IL-1β and IL-6 mRNA expression levels were compared between groups.

2.8.1 | RNA extraction from lung tissue

Lung samples were individually weighed and stored in RNAlater RNA Stabilization Reagent (Qiagen) at +4 C for up to 4 weeks. RNA isolation steps of the individually weighed tissue samples were carried out using the RNeasy Mini Kit (Qiagen) in the Qiaqube RNA isolation device (Qiagen) according to the manufacturer's recommendations. Total mRNA amount was measured at 260 nm with Nanodrop spectrophotometry (EPOCH, Biotek).

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2.8.2 | Reverse transcriptase reaction and cDNA synthesis

cDNA synthesis was performed from total RNA using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit enzyme. Each reaction was performed with 10 μL RNA, and cDNA synthesis was provided with the Applied Biosystems Veriti 96 Well Thermal Cycler according to the following temperature values. The amount of cDNA was determined by measurement with Nanodrop spectrophotometry (EPOCH Plate, Biotek) and stored at −20°C.

2.8.3 | Quantitative determination of mRNA expression by real-time PCR

Relative mRNA expression analyses of TNF-α, IL-1β and IL-6 from cDNA obtained from mouse lung tissue RNA were performed with the Taqman Gene Expression kit in the StepOne Plus Real-Time PCR device (Applied Biosystems). Real-Time PCR was performed with a method using primers specifically prepared for mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. For each tissue, 9 μL cDNA (100 ng), 1 μL Primer Perfect Probe mixture and 10 μL QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany) were used, and the total volume was adjusted to 20 μL in each measurement. According to the procedure, the plates of the Applied Biosystems StepOnePlus Real Time PCR device were heated at 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 15 s at 94°C and 60 s at 60°C were applied. All data were expressed as fold change in expression compared to the control (healthy) group using the $2^{-\Delta\Delta C_t}$ method.[21]

2.9 | Histopathological Analyses

Lung tissue samples taken from mice were fixed in 10% formaldehyde. After routine follow-up of the tissues, 4 μm thick sections were taken after embedding in paraffin. After deparaffinisation and rehydration procedures, they were stained with haematoxylin–eosin (H&E). All parts of the tissues were examined using a light microscope (Nikon Eclipse 80i), and then photographic images of the slides were taken using a digital camera. Sections obtained from lung tissue were evaluated for alveolar structure, alveolar wall thickness, oedema, haemorrhage, inflammation, increased capillary congestion and capillary permeability, haemorrhage, oedema and congestion in the parenchyma.[22]

2.10 | Statistical Analyses

IBM SPSS 20 statistical analysis program and GraphPad Prism 8.0 program were used for statistical analysis of ELISA findings, PCR findings and antioxidant measurement results. The normality distribution of continuous variables was examined using the Shapiro Wilk-W test and Kolmogorov Simirnov test. ANOVA test was preferred in cases where the normal distribution condition was met, and Kruskal–Wallis test was preferred in cases where it was not met in the comparison of continuous variables with three or more independent groups. Post-hoc tests after ANOVA test were performed using Tukey test when variances were homogeneous, and Tamhane's T2 test when variances were not homogeneous. The level of statistical significance was determined as $p < .05$.

3 | RESULTS

3.1 | Serum and tissue IL-1β, IL6, TNF-α, MDA levels

In ELISA measurements made from serum samples, statistically significant differences were found between groups in terms of IL-1β IL-1β, IL6, TNF-α and MDA levels ($p < .05$). Cytokine levels in the healthy control group were the lowest among all groups, and statistically significant differences were observed between it and all other groups ($p < .05$). When the sepsis group IL-1β IL-1β, IL6, TNF-α and MDA levels were examined, it was observed that cytokine levels were significantly increased compared to the healthy group; it was observed that it had the highest cytokine concentration ($p < .05$). When the treatment groups were examined, it was observed that the cytokine concentration decreased in a dose-dependent manner, and the sepsis+10 AVA group showed a statistically significant difference from the sepsis group ($p < .05$). (Figures 1 and 2).

3.2 | Impacts of AVA on oxidant levels and antioxidant lung tissue and serum enzyme activities

In biochemical measurements made from mice serum and lung tissues, statistically significant differences were found between groups in terms of GSH, CAT and SOD enzyme activities ($p < .05$). When the healthy group results were examined, it was observed that GSH, CAT and SOD enzyme activities were the highest among all groups, and statistically significant differences were

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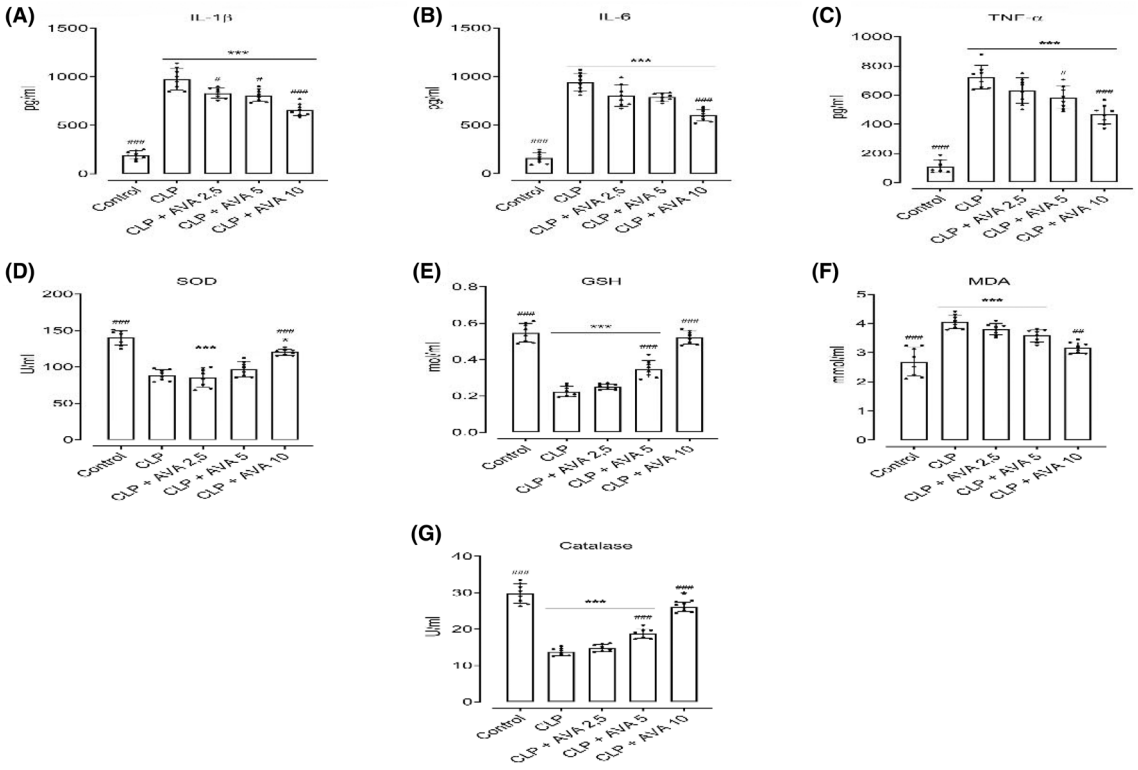

FIGURE 1 Serum IL-1β, IL6, TNF-α, SOD, GSH, MDA, Catalese levels. *$p < 0.05$ and ***$p < 0.001$ vs. control group. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. sepsis group.

observed between it and all other groups ($p < .05$). When the sepsis group GSH, CAT and SOD enzyme activities were examined, it was observed that GSH enzyme activities were significantly lower than the healthy group, and a significant difference was observed between the healthy group and sepsis+10 AVA groups ($p < .05$). When the treatment groups were examined, it was observed that GSH, CAT and SOD enzyme activities increased in a dose-dependent manner, and the sepsis+10 AVA group showed a statistically significant difference from the sepsis group ($p < .05$) (Figures 1 and 2).

3.3 | Lung tissue IL-1β, IL6, TNF-α relative mRNA expression measurement results

In PCR gene expression analysis performed from lung tissues, statistically significant differences were found between groups in terms of IL-1β, IL6 and TNF-α levels ($p < .05$). When the healthy group results were examined, it was observed that IL-1β, IL6 and TNF-α expression levels were the lowest among all groups, and statistically significant differences were observed between it and all other groups ($p < .05$). When the sepsis group cytokine mRNA expression levels were examined, it was observed that expression levels were significantly increased compared to the healthy group and treatment groups ($p < .05$). When the treatment groups were examined, it was observed that expression levels decreased in a dose-dependent manner ($p < .05$) (Figure 3).

3.4 | Histopathological analyses

Normal bronchioles, bronchi and normal lung parenchyma were observed in the lung tissue sections of the healthy control group. In the sepsis group, significant perivascular inflammation, perivascular oedema, interstitial inflammation and oedema and pathological changes such as hyaline membrane were observed. Dose-dependent histopathological improvements were observed in the treatment groups, and minimal pathological changes were observed, especially in the AVA 10 mg/kg group. The histopathological findings of the examined lung tissues are presented in Figure 4:

- Group 1: Healthy control group: Healthy lung parenchyma, normal bronchiole, respiratory bronchiole, alveolar sac and normal vascular structures were observed.
- Group 2: Sepsis group: Significant perivascular inflammation, perivascular edema, interstitial inflammation and edema, hyaline membrane was observed.
- Group 3: CLP + AVA 2.5 mg/kg: Interalveolar inflammation, hyaline membrane formation was observed.

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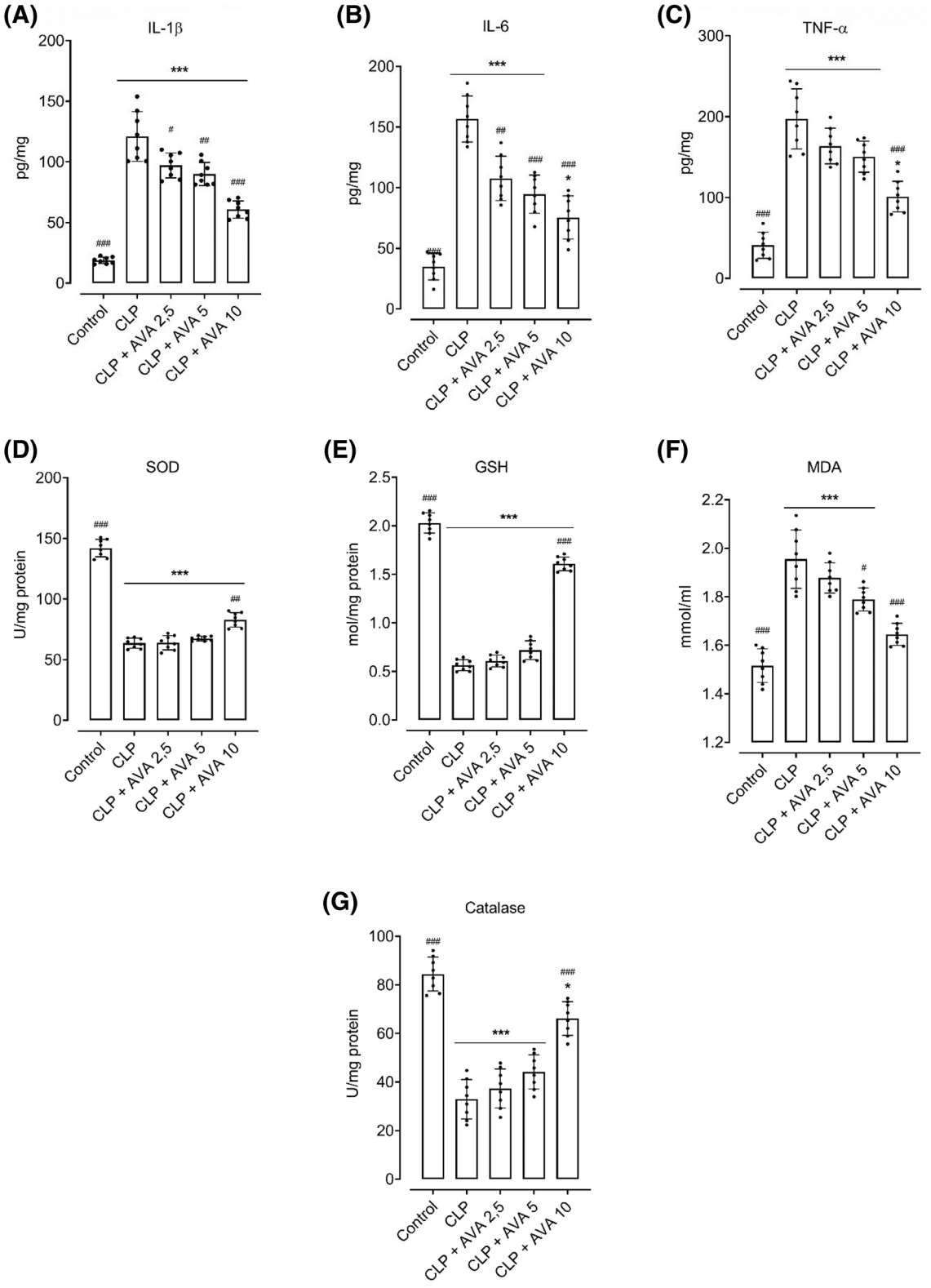

FIGURE 2 Lung tissue IL-1β, IL6, TNF-α, SOD, GSH, MDA, Catalese levels. *$p < 0.05$ and ***$p < 0.001$ vs. control group. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. sepsis group.

- Group 4: CLP + AVA 5 mg/kg: Peribronchial inflammation, interalveolar inflammation was observed.
- Group 5: CLP + AVA 10 mg/kg: Interalveolar spaces, minimal interalveolar thickening was observed.

4 | DISCUSSION

Sepsis is a life-threatening organ failure that occurs as a result of the host's dysregulated response to infection. The

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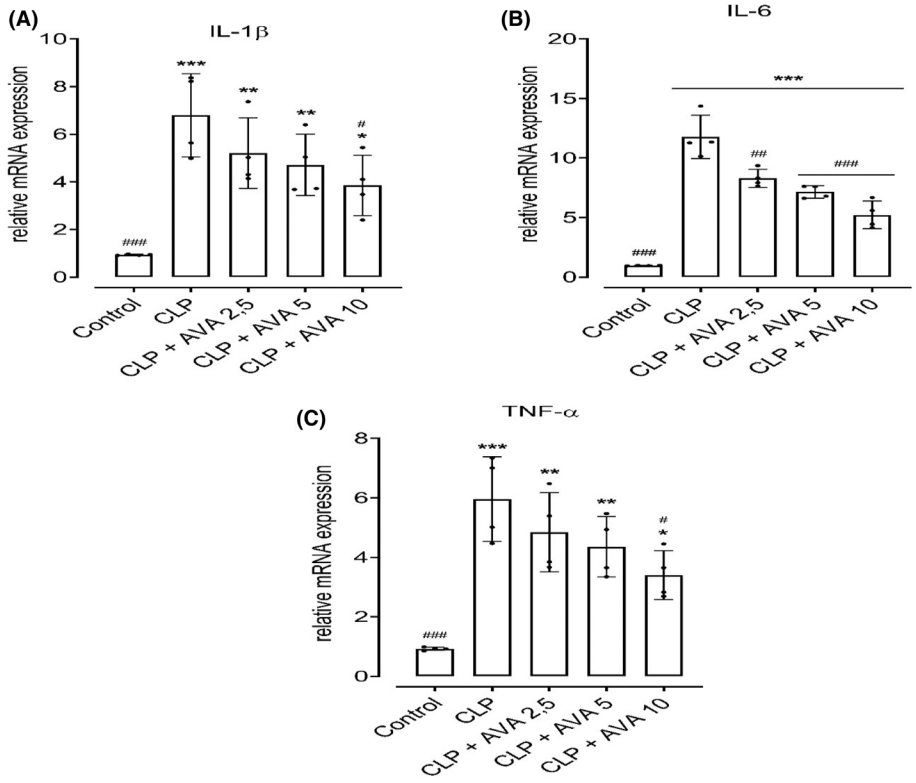

FIGURE 3 Lung tissue IL-1β, IL6, TNF-α relative mRNA expression measurement results. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. sepsis group.

lungs are the first and most affected organs in sepsis. Acute lung injury (ALI) occurs as a pulmonary manifestation of the inflammatory process due to acute damage to the pulmonary vascular endothelium, increased capillary permeability and leakage into the interstitial space.[1,23] In preclinical studies, this process has been explained by various experimental models. Bacterial endotoxin injection (lipopolysaccharides), bacterial culture infusion and disruption of the protective barrier, CLP, are the most commonly used research methods. Among these methods, CLP is accepted as the gold standard model that best reflects human immune reactions in abdominal sepsis.[24,25] In our study, due to the central role of the lungs in sepsis, the effects of sepsis on lung tissue were examined in detail by creating experimental sepsis with CLP.

Although there are supportive approaches in the management of sepsis, there is no specific and widely accepted primary treatment directly targeting the pathophysiology of sepsis.[1,26] In order to treat the disease, the pathophysiology of the disease must be well understood. Biomarkers are the most commonly used tools in the diagnosis, follow-up and treatment of the disease. IL-1β, IL6, TNF-α are the leading markers.[26,27] These mediators are cytokines involved in the initial response to inflammation. TNF-α and IL-1 both activate endothelial cells and attract polymorphonuclear (PMN) leukocytes to the site of inflammation, while IL-6 increases the production of acute phase reactants and induces cells in the bone marrow to produce PMNs. For these reasons, these three cytokines serve as biomarkers of sepsis.[5,27,28] In our study, the significant increase observed in TNF-α, IL-1β and IL-6 levels in serum and lung tissue clearly supports the critical role of these cytokines in the pathogenesis of sepsis. These findings are also consistent with previous research.[29]

Oxidative stress is one of the basic stages of sepsis pathophysiology. MDA is an important end product of lipid peroxidation and a commonly used biomarker of oxidative stress. The increase in MDA levels indicates increased oxidative stress and therefore cell damage.[30] In our study, MDA levels were significantly increased in the sepsis group at serum and tissue levels as expected, and it was shown that they gradually decreased in the treatment groups in a dose-dependent manner. The decrease in antioxidant enzymes in sepsis reduces the body's capacity to counteract the increased ROS production and neutralise oxidative damage.[8,31] This is an important factor contributing to the progression of sepsis and the development of organ dysfunctions. Increased oxidant levels cause endothelial dysfunction, leading to sepsis-related lung injury.[32] SOD, CAT and GPx are antioxidant enzymes that form the basic defence line against free radicals in the cell.[9] They make superoxide and hydrogen peroxide less harmful. These antioxidants play a role in the control of oxidative stress, inflammation and redox regulation.[33]

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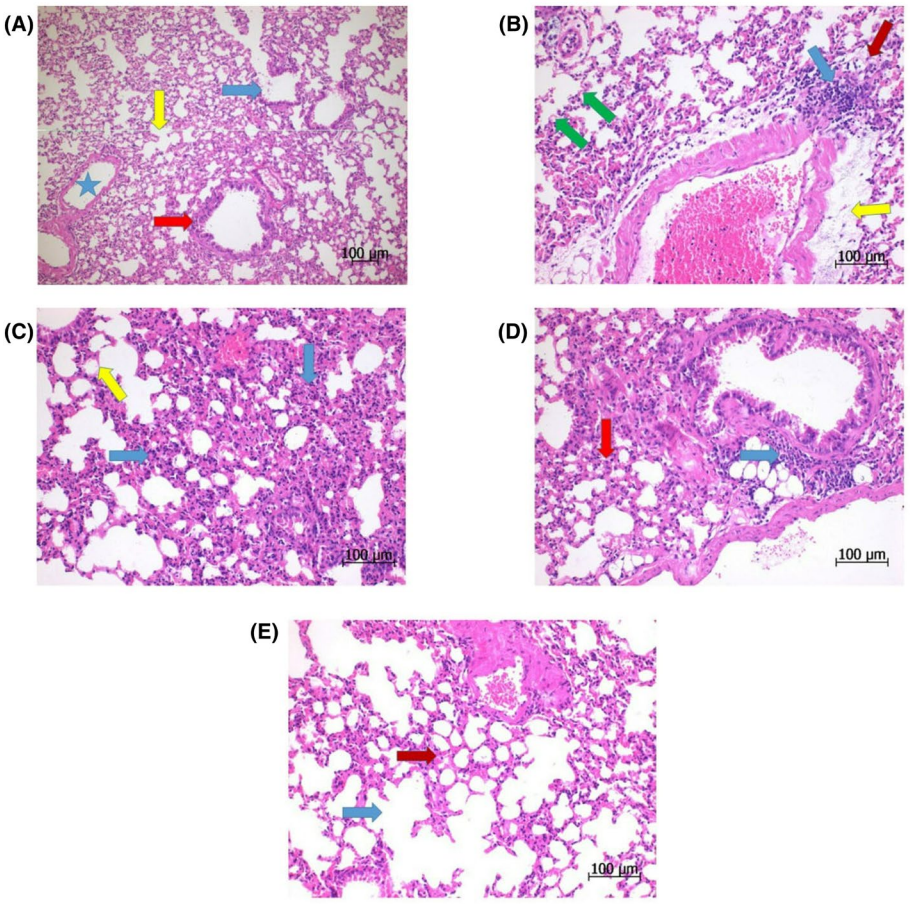

FIGURE 4 Examination of a histopathological view in lung tissue. (A) (Group 1: Healthy control group) healthy lung parenchyma; Red arrow: Bronchiole, Blue arrow: Respiratory bronchiole, Yellow arrow: Alveolar sac, Star: Vascular structure. (B) (Group 2: CLP Group (Sepsis)), Blue arrow: Perivascular inflammation, Yellow arrow: Perivascular edema, Red arrow: Interstitial inflammation and edema, Green arrow: Hyaline membrane. (C) (Group 3: CLP + AVA 2.5 mg/kg), Blue arrows: Interalveolar inflammation, Yellow arrow: Hyaline membrane. (D) (Group 4: CLP + AVA 5 mg/kg), Blue arrow: Peribronchial inflammation, Red arrow: Interalveolar inflammation. (E) (Group 5: CLP + AVA 10 mg/kg), Blue arrow: Intraalveolar spaces, Red arrow: Minimal interalveolar thickening.

In recent studies, while the blood and tissue levels of these enzymes decrease in the sepsis group, the enzyme levels increase in the healthy group or groups with antioxidant drugs added.[29,34,35] In our study, the effect of these three antioxidant enzymes was discussed. Parallel to the literature, enzyme activities were lowest in the serum and tissues of the experimental groups in which sepsis was induced, while they were highest in the sham groups and groups with antioxidant drugs added. Many drugs with SOD mimetic effects have been found to be effective on sepsis.[33,36,37] Avasopasem manganese has been shown to have a healing effect on radiation-induced mucositis in head and neck cancers with its superoxide scavenging activity (Phase II b clinical study). Superoxide anion (O_2^-) production increases after radiation, and superoxide radicals are reduced by the antioxidant effect of AVA.[38] AVA also exhibits an antitumor effect through a hydrogen peroxide-dependent mechanism by interacting synergistically with high-dose radiation regimens.[39,40] Manganese superoxide dismutase overexpression eliminates the overexpression of catalase and glutathione peroxidase, which metabolise H_2O_2, and exerts an antitumor effect.[39,40] There are also Phase 2 studies on the effect of AVA on the current disease, covid 19.[12]

In our study, avasopasem manganese treatment significantly reduced lung damage in the experimental sepsis model. A significant dose-dependent decrease in inflammatory cytokine levels and a significant dose-dependent increase in antioxidant enzyme levels were observed in the AVA-treated groups. These findings suggest that AVA may exhibit significant therapeutic effects by effectively modulating inflammation and oxidative stress processes that play a role in sepsis pathogenesis. Histopathological analyses in our study strongly support the protective effect of AVA on lung tissue. While widespread acute lung injury findings such as significant inflammation, edema, thickening of alveolar walls and hyaline membrane formation

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were observed in the lung tissue of the sepsis group, it was observed that this damage was significantly reduced and tissue structure was significantly improved in the AVA-treated groups. Especially in the high-dose AVA-treated group (10 mg/kg), the lung tissue was observed to be close to normal histological structure. These histopathological findings are also consistent with biochemical and molecular level results and support that AVA has a strong protective effect against sepsis-induced lung injury.

In our study, a significant increase in mRNA expression levels of pro-inflammatory cytokines in lung tissue was observed in the CLP-induced sepsis model. In particular, IL-1β, IL-6 and TNF-α mRNA expression levels were significantly higher in the sepsis group compared to the control group. These findings indicate that the transcription of cytokine genes increases in the early stages of sepsis, and therefore the production of these cytokines accelerates.[5,27,28] In the groups treated with avasopasem manganese, a dose-dependent decrease in cytokine mRNA expression levels was detected. Especially in the high-dose AVA (10 mg/kg) treated group, the expression levels of cytokines were significantly lower than in the sepsis group. These results suggest that AVA exerts an anti-inflammatory effect not only by modulating protein levels of cytokines but also by modulating gene expression. AVA may be affecting transcriptional mechanisms that play an important role in the pathogenesis of sepsis and therefore may exhibit a broader therapeutic effect. For example, some studies have shown that SOD mimetics reduce the expression of inflammatory genes by inhibiting the activity of transcription factors such as Nuclear Factor kappa B.[13,41] However, the precise mechanism of action of AVA at the transcriptional level has not been fully elucidated in our study. Future studies should investigate in more detail which transcription factors are affected by AVA and how it regulates the expression of inflammatory genes.

Our study has certain strengths and limitations. The most important strength is that it is the first study to investigate the effect of AVA on sepsis and has shown the protective effect of this drug on sepsis. The most significant limitation of our study is that it is an experimental study and has not yet been clinically applied. Secondly, our study evaluated only the short-term effects of AVA. There is insufficient information about the long-term effects and potential side effects of AVA. Finally, the number of animals used in our study is limited due to the cost burden of the drug. Our findings need to be confirmed with larger-scale and randomised controlled studies, and the clinical potential of AVA needs to be better understood.

In conclusion, in the CLP-induced sepsis model, there was a significant increase in the levels of inflammatory cytokines (IL-1β, IL-6, TNF-α) and MDA, an important marker of oxidative stress, in lung tissue, while the levels of antioxidant enzymes (SOD, CAT, GSH) decreased significantly. Also, while the mRNA expression levels of cytokines increased significantly in the sepsis group, they decreased in a dose-dependent manner in the treatment group. More importantly, in the groups treated with AVA, these pathological changes were partially or significantly reversed, and especially high-dose AVA (10 mg/kg) administration was observed to have a significant protective effect by suppressing the sepsis-induced inflammatory response and increasing the antioxidant capacity of lung tissue. However, in order to translate these preclinical findings into clinical practice, more comprehensive and randomised controlled clinical trials are needed to determine the efficacy, safety and optimal dose of AVA in different types of sepsis.

AUTHOR CONTRIBUTIONS

P.R.: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing-original draft, writing-review& editing, visualization, supervision, project administration. A.E.: Investigation, Resources, data curation, Writing-Original Draft, Writing-review& Editing, Visualization, Supervision. T.T.: Conceptualization, methodology, software, formal analysis, investigation, resources, data curation, writing-original draft, visualization, project administration. S.T.A.: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing-original draft, writing-review& editing, visualization, supervision, project administration, K.Z.: Conceptualisation, methodology, software, formal analysis, investigation, resources, data curation, writing–original draft, visualisation, project administration. Ö.S.: Conceptualisation, methodology, investigation, resources, data curation, writing–original draft, visualisation, project administration. A.V.: Conceptualisation, methodology, investigation, resources, data curation, writing–original draft, visualisation, project administration. H.Z.: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing-original draft, writing-review& editing, visualization, supervision, project administration. A.S.S.: Investigation, Resources, data curation, Writing-Original Draft, Writing-review& Editing, Visualization, Supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author.

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