Exploring the anti-inflammatory activity of boron compounds through the miR-21/PTEN/AKT pathway in cecal ligation and puncture-induced sepsis

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Abstract. The present study investigated the impact of boric acid (BA) and borax (BX) on markers of inflammation and modifications in miR-21/PTEN/AKT pathway genes in the liver and kidney tissues of Sprague Dawley male rats with sepsis induced by cecal ligation and puncture (CLP). A total of 60 male Sprague Dawley rats were randomly divided into 6 groups, each containing 10 animals as follows: Control, CLP (where the model was created), 20 mg/kg BX (CLP + BX1), 40 mg/kg BX (CLP + BX2), 20 mg/kg BA (CLP + BA1) and 40 mg/kg BA (CLP + BA2). Liver and kidney tissues were analyzed for histopathological changes, immunopositivity for tumor necrosis factor-α, interleukin (IL)-6 and IL-10, and gene expression of microRNA-21 (miR-21), phosphatase and tensin homolog (PTEN) and AKT. Gene expression analysis in the liver tissues revealed a significant decrease in miR-21, and a marked but not significant decrease in PTEN levels in the CLP group, while AKT expression was significantly increased in the CLP group, and was significantly decreased in CLP + BA1 group compared with in the CLP group. In the kidney tissues, miR-21 levels were significantly decreased in the CLP group, but the CLP + BA2 group showed a significant increase compared with in the CLP group. These results suggest the potential therapeutic benefits of low-dose BA and BX in ameliorating sepsis-induced tissue damage, emphasizing the need for further exploration of their mechanisms of action.

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Introduction

Sepsis appears as a result of the host's inadequate and irregular response to an infection, leading to the disruption of critical organ functions. Sepsis is a life-threatening condition characterized by a series of pathophysiological symptoms that may progress to septic shock, and associated with an elevated risk of death due to extreme cellular, metabolic and circulatory abnormalities (1). The increasing occurrence and complex clinical presentation of both sepsis and septic shock pose significant challenges and burdens for emergency physicians (2,3). Since the initial consensus definition in 1991, the prevalence of sepsis and septic shock has continued to rise, with an estimated 49 million sepsis cases and 11 million sepsis-related deaths reported worldwide in 2017 (4,5). Consequently, the World Health Organization has recognized sepsis as a critical global health concern (3). Several factors contribute to the occurrence of sepsis, including the higher mean age of patients, the increasing use of invasive procedures, the administration of immunosuppressive drugs and chemotherapy, and the emergence of antibiotic resistance. Despite advances in treatment approaches,

individuals affected by sepsis remain at risk of experiencing fatal outcomes during their hospital stay (6).

In severe conditions such as sepsis, the organs most often impacted are the kidneys, liver, lungs and heart, as well as the central nervous and hematological systems (7). The liver holds a central position in maintaining metabolic and immunological balance, and can suffer harm from pathogens, toxins or inflammatory agents, making it a critical organ requiring prompt treatment for survival (8). During an episode of sepsis, liver injuries manifest through a series of stages, ranging from impaired liver cell function to liver damage and eventual liver failure. Liver failure, the most severe form of damage, is characterized by the loss of 80-90% of liver cell functionality. Identifying liver dysfunction and failure directly is a crucial factor for the successful resolution of a sepsis episode (7,9,10). Renal dysfunction emerges as another focal point in the progression of sepsis and is the predominant factor contributing to acute kidney injury (AKI) in critically ill individuals (11). The risk of in-hospital mortality among patients experiencing sepsis-related AKI is >60%, surpassing by far the risks faced by patients with sepsis but without AKI and those with AKI unrelated to sepsis (12). The precise mechanisms behind AKI have been partially identified, yet the primary focus is on the onset of renal hypoperfusion. Current evidence, however, indicates that local microcirculation and inflammatory signals, including ischemia-reperfusion injury, oxidative stress and tubular apoptosis, assume an even more pivotal role (13).

In the development of sepsis, inflammation plays a vital yet intricate role (14). The uncontrolled escalation in the release of pro-inflammatory cytokines by the immune system can result in a dysregulated and exaggerated cytokine storm that leads to vasodilation, increased vascular permeability and a decrease in blood pressure, adversely affecting blood flow to the vital organs (15). Moreover, it disrupts the normal functioning of the blood clotting system, culminating in disseminated intravascular coagulation (16). These uncontrolled inflammatory responses may lead to widespread organ dysfunction and failure, thereby defining the severity of the sepsis. To date, several contributing biomarkers, genes and pathways have been investigated for their role in the escalating cytokine storm that may lead to sepsis (17). Among others, small non-coding RNAs such as microRNAs (miRNAs/miRs) have emerged as key regulators over the years due to their role in inflammatory gene expression at multiple levels, and their potential as biomarkers and therapeutic targets in controlling abnormal inflammatory reactions in the body (18,19). miR-21 has been found to play a role in inflammation and sepsis. Studies have shown that miR-21 expression is upregulated in sepsis-induced acute lung injury (ALI) and acute liver injury (20,21). In septic mice, miR-21 expression is enhanced in peritoneal macrophages and neutrophils, and its deletion in myeloid cells leads to improved survival, decreased bacterial growth, and reduced systemic inflammation and organ damage (21,22). The protective effect of miR-21 in sepsis-induced ALI is mediated by regulating phosphatase and tensin homolog (PTEN) (23). PTEN negatively regulates the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which alters inflammatory responses through the release of inflammatory factors, and the recruitment and activation of immune cells (24,25).

Boron compounds are essential micronutrients for plants, and while they are not classified as essential nutrients for humans, they seem to play various roles in plant and animal physiology (26). Although they are present in trace amounts in naturally derived food products and historically have been used as food preservatives, such as during the 1910s to prevent spoilage during World War I, their use in medicine is limited and mostly as adjuvants. Boric acid (BA; chemical formula: H_3BO_3), known by various names such as hydrogen borate, boracic acid or orthoboric acid, is recognized as a derivative of a weak monobasic acid. Borax (BX; chemical formula: $Na_2B_4O_7{\cdot}10H_2O$), also referred to as sodium borate, sodium tetraborate decahydrate or disodium tetraborate, is a naturally occurring mineral. Boron compounds are commonly employed as adjuvants with antiseptic properties in ophthalmological and, for limited purposes, in dermatological products (BA) or as a precursor for various chemical compounds (27-30). Compounds containing boron, such as BA or BX, have demonstrated a broad spectrum of biological activities, including antibacterial, antiviral, antifungal, anti-carcinogenic, anti-invasive, anti-angiogenic, anti-mutagenic, anti-inflammatory and antioxidant properties (26,31). Previous studies suggest that boron induces changes in barrier function, proliferation and apoptosis in rat intestinal epithelial cells through the PI3K/AKT signaling pathway (32,33). Given the complex involvement of inflammation in sepsis, the present study focused on investigating the *in vivo* effects of BA and BX on inflammation biomarkers, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10, and on alterations in miR-21/PTEN/AKT pathway genes within the liver and kidney tissues of rats experiencing sepsis induced by cecal ligation and puncture (CLP).

Materials and methods

Animals. Sprague Dawley male rats (n=60), aged between 8 and 10 weeks and weighing 200-250 g, were utilized in this study. The animals were acquired from the Animal Shelter affiliated with Ataturk University's Medicinal and Experimental Application and Research Center (Erzurum, Türkiye). All procedures strictly adhered to the ethical guidelines sanctioned by the ethical committee, and the animal research used in the scientific procedures in the study was conducted under the guidance of Directives 2010/63/EU, which regulate animal research in the European Union regarding the protection of animals used for scientific purposes. Approval for all procedures was granted by the Ethics Committee at Kastamonu University (Kastamonu, Türkiye; approval no. 28/4). The animals were comfortably housed in standard polypropylene cages within a meticulously controlled environment, maintained at a temperature of 22±1˚C, with relative humidity ranging between 50 and 60%, and adhering to a 12-h light/dark photoperiod. The animals were provided with ample quantities of standard food and tap water available *ad libitum* following the procedures outlined by the Animal Shelter at Ataturk University's Medicinal and Experimental Application and Research Center, with the animal feed being commercially supplied by the research center.

Experimental design: CLP-induced sepsis model. Sepsis was modelled through the CLP method, as previously

described (34). Rats underwent intraperitoneal injection for anesthesia, receiving a combination of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight). Upon achieving a profound level of anesthesia, the abdominal region was depilated and a longitudinal midline incision (3 to 4 cm) was made to expose the cecum. Following a meticulous dissection of the cecal mesentery, a ligature was applied at the predetermined position to achieve the desired severity grade, effectively occluding the cecal lumen. Using an 18-gauge needle, four holes (two on each side) were created along the mesentery, extending from the distal end of the cecum to the ligated point, facilitating the milking of the cecum. The induction of abdominal sepsis ensued, with the bacterial flora from the stool emerging through the perforations in the milked cecum. Subsequently, the cecum was reintroduced into the peritoneal cavity, and 0.5 ml of normal saline was administered through the peritoneal cavity to all animals before suturing the incision. Closure of the abdominal incision occurred in two layers using 3.0 silk sutures. Following the experimental procedures, the rats were returned to their cages. The animals had unrestricted access to both food and water. A sepsis model induced by mid-grade CLP using an 18-gauge needle was established, with significant sepsis-associated changes observed around the 16th hour post-procedure, as indicated by Hubbard *et al* (35).

Experimental design: Boron administration. Six groups, each consisting of 10 rats, were established for the study with the following interventions: i) Control group, which did not receive any intervention or CLP sepsis induction; ii) CLP group, in which the sepsis model was applied, but no interventions were made; iii) CLP + BX1 group, which was treated with 20 mg/kg BX; iv) CLP + BX2 group, which was treated with 40 mg/kg BX; v) CLP + BA1 group, which was treated with 20 mg/kg BA; and vi) CLB + BA2 group, which was treated with 40 mg/kg BA. The control group and the treatment groups (CLP + BX1, CLP + BX2, CLP + BA1 and CLP + BA2) received intraperitoneal normal saline, with the treatment groups receiving BX (cat. no. 1303-96-4; MilliporeSigma) or BA (cat. no. 10043-35-3; MilliporeSigma) dissolved in normal saline 12 h after the induction of sepsis. The CLP group did not receive any treatment, serving as the untreated sepsis model.

Sacrifice and sample collection. The animals were humanely euthanized via intraperitoneal administration of ketamine at a dosage of 300 mg/kg combined with xylazine at a dosage of 30 mg/kg, and their vital signs were observed until their heartbeats ceased. Euthanasia was performed 24 h after sepsis induction, and tissue samples from the kidney and liver were collected for analysis. The kidney and liver tissues were fixed in a 10% formalin solution at room temperature for 24 h to ensure adequate preservation for subsequent histopathological and molecular analyses.

Gene expression analysis. The extraction of total RNA from paraffin-embedded kidney and liver tissues was performed using the High Pure FFPET RNA Isolation Kit (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's guidelines. Subsequently, cDNA synthesis was performed with the Second Strand cDNA Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, utilizing a consistent quantity of RNA from each sample. All primers (5'-3') used were purchased as TaqMan Gene Expression Assays (Table I). The expression data for β-actin and U6 in each tissue were used as the endogenous control (Table I). Gene expression levels were assessed through qPCR using the Light Cycler® 480 II with Light Cycler 480 Probes Master (Roche Diagnostic GmbH). Each animal's tissue was examined in triplicate. The annealing temperature for all gene regions was set at 60˚C, qPCR was performed in triplicate for each sample using the following conditions: Denaturation at 95˚C for 10 min, followed by 45 cycles of amplification at 95˚C for 10 sec, 60˚C for 30 sec, 72˚C for 1 sec and cooling at 40˚C for 10 sec. Reaction mixture including no cDNA used as negative control. and the gene expression levels were calculated as using the $2^{-\Delta\Delta Cq}$ method. b-actin and U6 were used as reference genes for the calculation of relative mRNA and miRNA expression levels, respectively (36,37).

Pathological analyses

Histopathology. Liver and kidney tissues obtained from rat necropsies were preserved in a 10% neutral formalin solution. Following routine alcohol-xylene processing, the tissues were embedded in paraffin blocks. Sections (4-μm thick) were placed on slides and stained with hematoxylin and eosin for 10 min at room temperature, and a semi-quantitative assessment of microscopic changes was conducted, with categorization as follows: No changes (score 0), mild (score 1), moderate (score 2), severe (score 3) and very severe (score 4).

Immunohistochemistry. For immunohistochemical evaluation, the 4-μm sections on the slides underwent de-paraffinization in xylene and alcohol, followed by a 10-min PBS wash and treatment with 3% H_2O_2, achieving endogenous peroxidase inactivation. Antigen retrieval was performed by subjecting the tissues to a 2x5-min treatment at 500 watts with an antigen retrieval solution (100X Citrate Buffer; cat. no. ab93678; Abcam). Subsequently, TNF-α (cat. no. SC-133192; Santa Cruz Biotechnology, Inc.), IL-6 (cat. no. DF6087; Affinity Biosciences) and IL-10 (cat. no. SC-8438; Santa Cruz Biotechnology, Inc.) primary antibodies were applied at a dilution of 1:200 and left to incubate overnight at 4˚C. For the secondary antibody step, the Large Volume Detection System: Anti-Polyvalent, HRP (cat. no. TP-125-HL; Thermo Fisher Scientific, Inc.), was used according to the manufacturer's instructions, typically involving incubation for ~45 min at room temperature. 3.3'-Diaminobenzidine served as the chromogen. After counterstaining with Mayer's hematoxylin for 1 min at room temperature, the slides were covered and examined under a light microscope. The percentages of immunopositivity detected in six random distinct fields were analyzed using the Fiji ImageJ program (License, GPLv3+; https://imagej.net/software/fiji/downloads). Two different pathologists independently evaluated the samples, scoring as follows: No reactivity (score 0), mild (score 1), moderate (score 2), severe (score 3) and very severe (score 4).

Statistical analysis. The disparities in molecular data among distinct groups were assessed through one-way analysis of variance (ANOVA) using IBM SPSS 20.0 statistical software (IBM Corp.). Variance uniformity within groups was

Table I. Primer sequences and annealing temperatures.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Temperature, ˚C	Assay ID
miR-21	TAGCTTATCAGACTGATGTTGA	GCCAGCACAGAATTAATACGAC	60	Rn04244285_s1
PTEN	AGAACAAGATGCTAAAAAGGACAA	TGTCAGGGTGAGCACAAGAT	60	Rn00477208_m1
AKT1	GTGGCAAGATGTGTATGAG	CTGGCTGAGTAGGAGAAC	60	Rn00583646_m1
β-actin	TGGTGGGTATGGGTCAGAAG	GACAATGCCGTGTTCAATGG	60	Hs99999903_m1
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT	60	001973

miR-21, microRNA-21; PTEN, phosphatase and tensin homolog.

confirmed using Levene's test, and normal distribution within each group was assessed through the Shapiro-Wilk test. Group differences in gene expression levels were established using one-way ANOVA, followed by a Tukey's post hoc test. For the statistical analysis of semiquantitative data determined histopathologically, intergroup differences were evaluated using the Kruskal-Wallis test followed by Dunn's post hoc test, and the group responsible for the variance was determined through the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Results

Histopathological findings. During the histopathological assessment of the liver and kidneys, differences were observed among the groups. Rats in the control group exhibited a normal histological appearance. By contrast, rats in the other groups showed histopathological findings, including necrosis, congestion and mononuclear cell infiltrations. The severity of necrosis and mononuclear cell infiltrations varied across groups, with the CLP group experiencing very severe effects, the CLP + BA1 group showing severe effects, the CLP + BA2 and CLP + BX1 groups displaying moderate effects, and the CLP + BX2 group exhibiting mild effects. The degree of congestion ranged from moderate in the CLP, CLP + BX2, and CLP + BA1 groups to mild in the CLP + BX1 and CLP + BA2 groups (Table II; Fig. 1).

The kidneys of rats in the control group exhibited normal histological features. Histopathologically, intertubular areas in the other groups showed signs of interstitial nephritis, attributed to mononuclear cell infiltrations. This microscopic finding was severe in the CLP and CLP + BX1 groups, moderate in the CLP + BX2 group, and mild in the CLP + BA1 and CLP + BA2 groups (Table III; Fig. 2).

Immunohistochemical findings. Immunohistochemically, significant differences were observed among the groups in terms of TNF-α, IL-6, and IL-10 staining in the liver and kidneys. In the liver tissues of control rats, there was no notable immunopositivity in the staining for TNF-α, IL-6 or IL-10. Varied levels of immunopositivity were detected in the other treatment groups. For TNF-α staining, the CLP group exhibited a very severe level, CLP + BX1 and CLP + BA1 groups showed a severe level, and the CLP + BX2 and CLP + BA2 groups displayed moderate immunopositivity. IL-6 staining revealed severe immunopositivity in the CLP group, a mild level in the CLP + BX2 group, and moderate in the other groups. In terms of IL-10 staining, the CLP + BX2 and CLP + BA2 groups exhibited mild immunopositivity, while the other groups showed moderate immunopositivity. Positive

Table II. Statistical evaluation of histopathological alterations in the liver tissues.

Groups	Necrosis	Congestion	Mononuclear cell infiltration
Control	0 (1.00)	0 (1.00)	0 (0.00)
CLP	4 (1.00)[a]	2 (0.00)[a]	4 (0.25)[a]
CLP + BX1	2 (1.00)[b]	1 (0.25)[b]	2 (0.25)[b]
CLP + BX2	1 (0.00)[c]	2 (0.00)[a]	1 (0.00)[c]
CLP + BA1	3 (0.00)[d]	2 (0.00)[a]	3 (0.00)[d]
CLP + BA2	2 (0.00)[b]	1 (0.00)[b]	2 (1.00)[b]

Values are represented as the median (IQR). [a-d]Signifies the variation among the groups (P<0.05). While the groups indicated with the same letter do not show statistical significance with each other, there is statistical significance at the level of P<0.05 between the groups with different letters. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

Table III. Statistical evaluation of histopathological alterations in the kidney tissues.

Groups	Interstitial nephritis
Control	0 (0.00)
CLP	3 (0.00)[a]
CLP + BX1	3 (0.25)[a]
CLP + BX2	2 (0.25)[b]
CLP + BA1	1 (0.00)[c]
CLP + BA2	1 (0.25)[c]

Values are represented as the median (IQR). [a-c]Signifies the variation among the groups (P<0.05). While the groups indicated with the same letter do not show statistical significance with each other, there is statistical significance at the level of P<0.05 between the groups with different letters. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

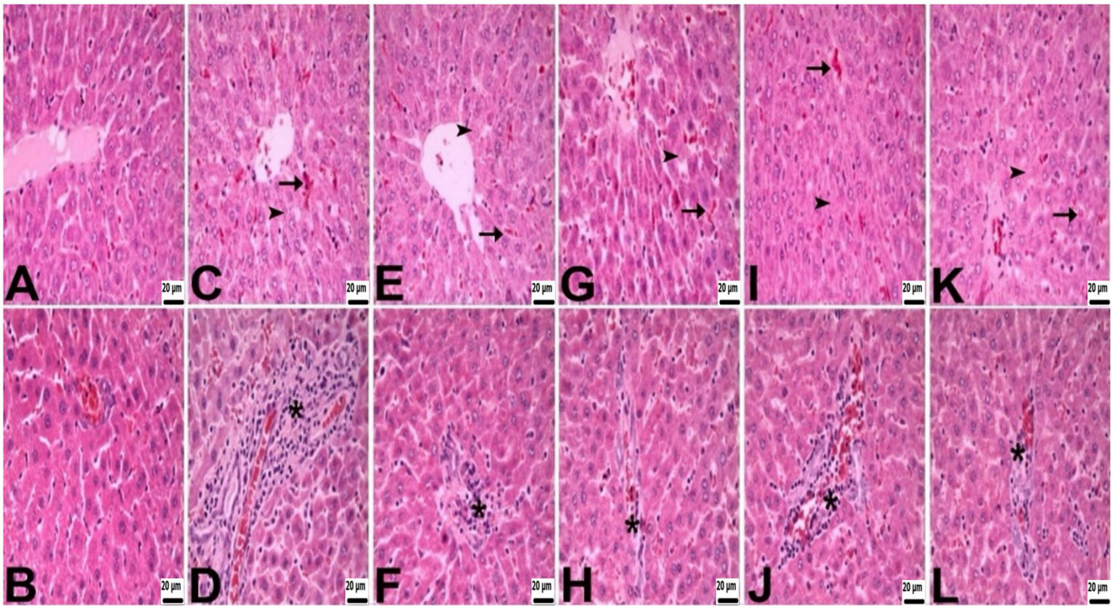

Figure 1. Hematoxylin and eosin staining of the liver tissues per group (n=10). (A and B) Control group: Normal histological appearance. (C and D) CLP group: Severe necrosis (indicated by the arrowhead) and mononuclear cell infiltration (indicated by the asterisk) with moderate congestion (indicated by the arrow). (E and F) CLP + BX1 group: Moderate necrosis (indicated by the arrowhead) and mononuclear cell infiltration (indicated by the asterisk) with mild congestion (indicated by the arrow). (G and H) CLP + BX2 group: Mononuclear cell infiltration (indicated by the asterisk) with moderate congestion (indicated by the arrow). (I and J) CLP + BA1 group: Mild necrosis (indicated by the arrowhead) and mononuclear cell infiltration (indicated by the asterisk) with moderate congestion (indicated by the arrow). (K and L) CLP + BA2 group: Severe level necrosis (indicated by the arrowhead) in specific areas with moderate necrosis (indicated by the arrowhead), mononuclear cell infiltration (indicated by the asterisk) and mild congestion (indicated by the arrow). CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

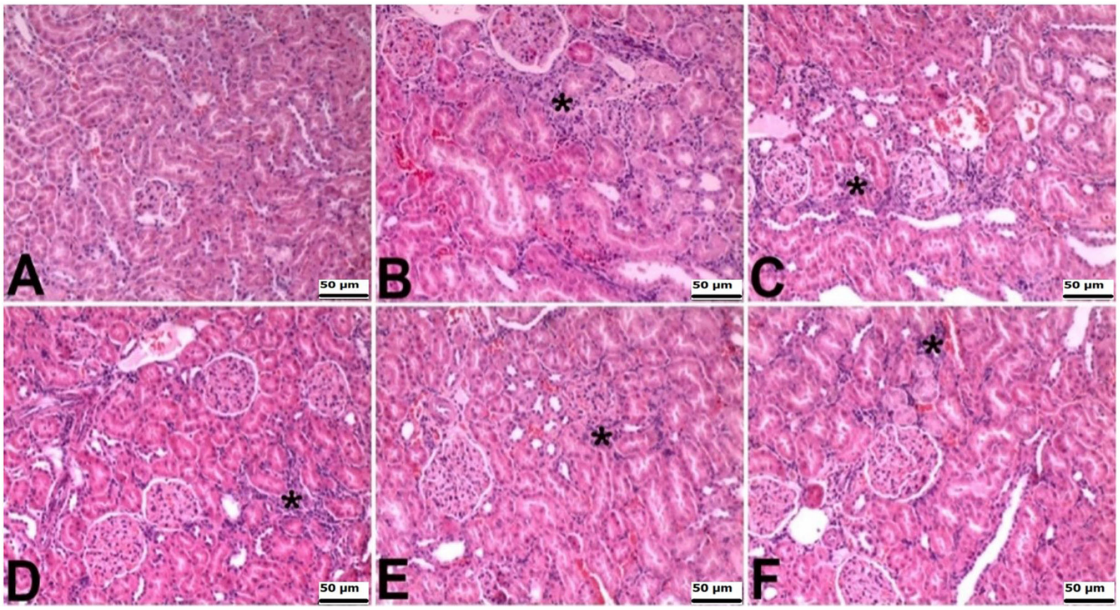

Figure 2. Hematoxylin and eosin staining of the kidney tissues per group (n=10). (A) Control group: Normal histological appearance. (B) CLP group: Severe interstitial nephritis (indicated by the asterisk). (C) CLP + BX1 group: Severe interstitial nephritis (indicated by the asterisk). (D) CLP + BX2 group: Inter-level interstitial nephritis (indicated by the asterisk). (E) CLP + BA1 group: Slight interstitial nephritis (indicated by the asterisk). (F) CLP + BA2 group: Slight interstitial nephritis (indicated by the asterisk). CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

immunohistochemical findings were localized intracytoplasmically in hepatocytes (Table IV; Figs. 3-5).

In the kidneys of control rats, there was no significant immunopositivity in terms of the staining for TNF-α, IL-6 or IL-10. Varied levels of immunopositivity were observed in the other groups. TNF-α immunopositivity was severe in the CLP and CLP + BX1 groups, moderate in the CLP + BX2 group, and mild in the CLP + BA1 and CLP + BA2 groups. IL-6 immunopositivity was very severe in the CLP group, severe in the CLP + BX1 and CLP + BX2 groups, and mild in the CLP + BA2 and CLP + BA1 groups. IL-10 immunopositivity was moderate in the CLP group and mild in all the other groups.

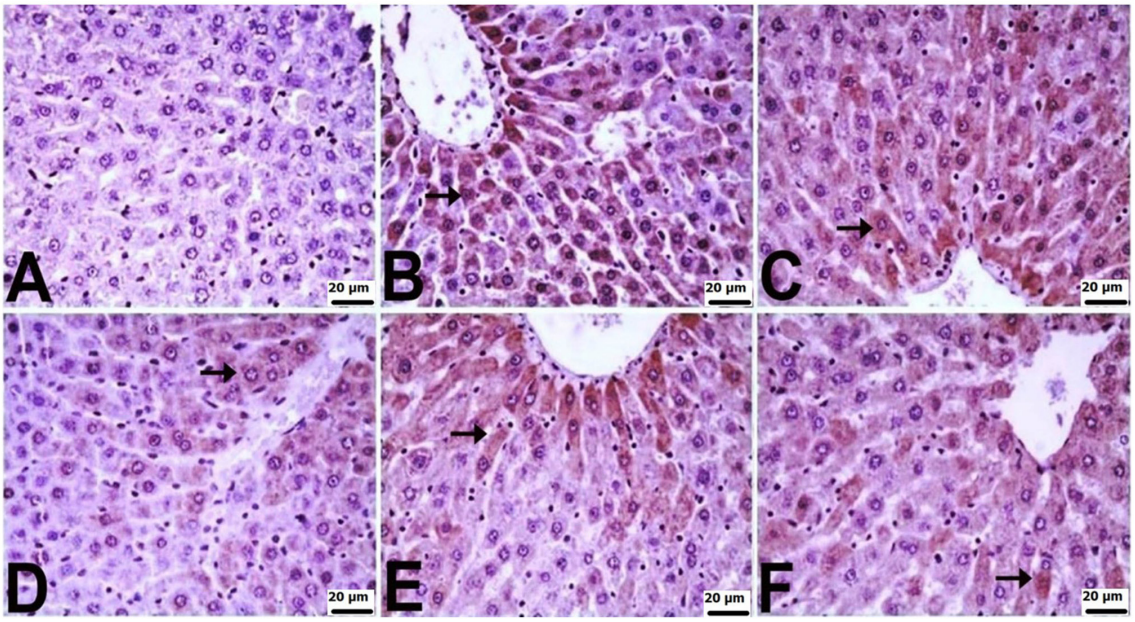

Figure 3. Immunohistochemical staining of tumor necrosis factor-α in the liver tissues per group (n=10). (A) Control group: Immunonegativity. (B) CLP group: Severe immunopositivity. (C) CLP + BX1 group: Moderate immunopositivity. (D) CLP + BX2 group: Moderate immunopositivity. (E) CLP + BA1 group: Severe immunopositivity. (F) CLP + BA2 group: Moderate immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

Table IV. Statistical assessment of immunopositivity in immunohistochemical staining conducted with TNF-α, IL-6 and IL-10 in the liver tissues.

Groups	TNF-α	IL-6	IL-10
Control	0 (1.00)	0 (0.25)	0 (0.00)
CLP	4 (1.00)^{a}	3 (0.25)^{a}	2 (0.00)^{a}
CLP + BX1	3 (0.25)^{b}	2 (0.00)^{b}	2 (0.00)^{a}
CLP + BX2	2 (1.00)^{c}	1 (0.25)^{c}	1 (0.00)^{b}
CLP + BA1	3 (1.00)^{b}	2 (0.00)^{b}	2 (0.25)^{a}
CLP + BA2	2 (0.25)^{c}	2 (0.00)^{b}	1(0.25)^{b}

Values are represented as the median (IQR). $^{a-c}$Signifies the variation among the groups ($P<0.05$). While the groups indicated with the same letter do not show statistical significance with each other, there is statistical significance at the level of $P<0.05$ between the groups with different letters. CLP, cecal ligation and puncture; BA, boric acid; BX, borax; TNF-α, tumor necrosis factor-α; IL, interleukin.

TNF-α and IL-10 immunopositivity was observed in tubular epithelial cells, while IL-6 immunopositivity was observed in both tubular epithelial cells and the glomerulus (Table V; Figs. 6-8).

Gene expression analysis. The changes in miR-21 expression levels in the liver tissue between the control, CLP and various treatment groups are presented in Fig. 9A. In the control group, miR-21 expression levels were observed at baseline, while a significant reduction was detected in the CLP group. Similarly, in the CLP + BA1 and CLP + BA2 treatment groups, miR-21 expression levels remained low, showing a profile comparable to the CLP group. By contrast, in the CLP + BX1 treatment group, a significant increase in miR-21 expression levels was observed compared with those in both the control and CLP groups The CLP + BX2 treatment group also showed a tendency to increase miR-21 levels, but this increase was not statistically significant. These findings indicated that in liver tissue, the CLP model may suppress miR-21 expression, while BX1 treatment effectively reverses this suppression. The enhancing effect of BX1 on miR-21 expression suggests a potential regulatory role in inflammation and related signaling pathways.

The mRNA expression levels of PTEN in the liver tissue across the control, CLP and treatment groups are shown in Fig. 9B. In the control group, PTEN expression levels were observed to be the highest, indicating its normal physiological activity under baseline conditions. By contrast, the CLP group exhibited a marked reduction in PTEN expression, suggesting a suppression of PTEN activity in response to the CLP-induced inflammatory state. Notably, treatment with BA1 and BA2 did not significantly restore PTEN expression, as the levels remained comparable to the CLP group; however, BX1 and BX2 treatments showed a tendency to increase PTEN expression, although these changes were not statistically significant.

The mRNA expression levels of AKT in the liver tissue across the control, CLP and treatment groups are shown in Fig. 9C. In the control group, AKT expression was observed at baseline levels. In the CLP group, a significant increase in AKT expression was detected compared with that in the control group, highlighting the activation of AKT in response to the CLP-induced inflammatory process. By contrast, treatment with BA1 resulted in a significant reduction in AKT expression compared with in the CLP group, suggesting that BA1 may suppress AKT activation. However, BA2, BX1 and BX2 treatments showed no significant differences compared with the CLP group, with AKT expression levels remaining elevated.

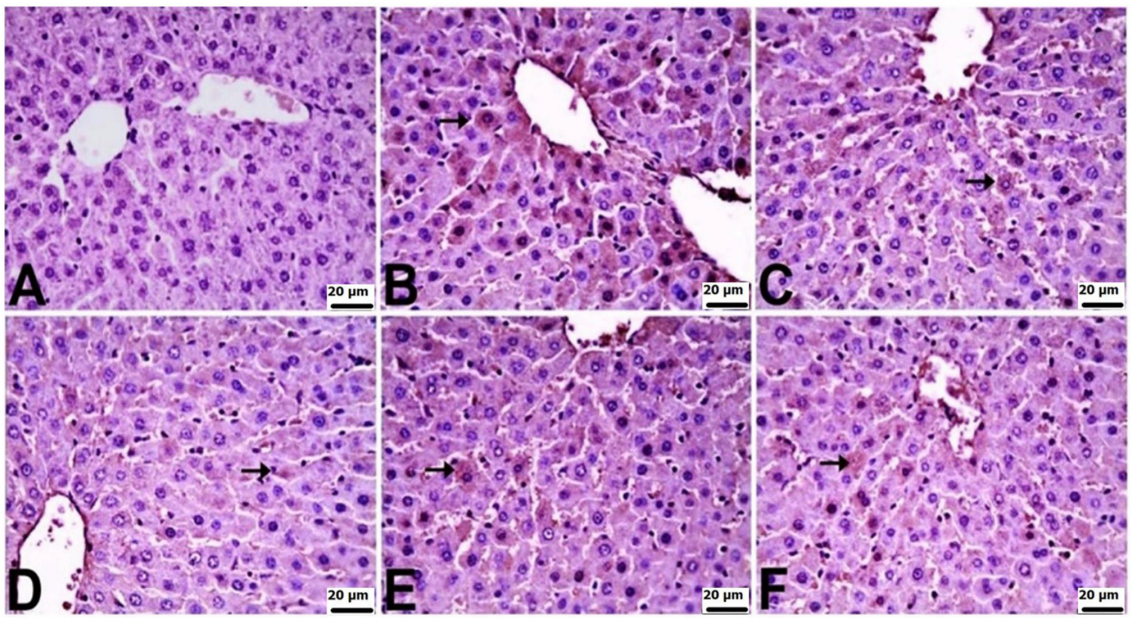

Figure 4. Immunohistochemical staining of interleukin-6 in the liver tissues per group (n=10). (A) Control Group: Immunonegativity. (B) CLP group: Severe immunopositivity. (C) CLP + BX1 group: Middle level immunopositivity. (D) CLP + BX2 group: Light immunopositivity. (E) CLP + BA1 group: Middle level immunopositivity. (F) CLP + BA1 group: Moderate immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

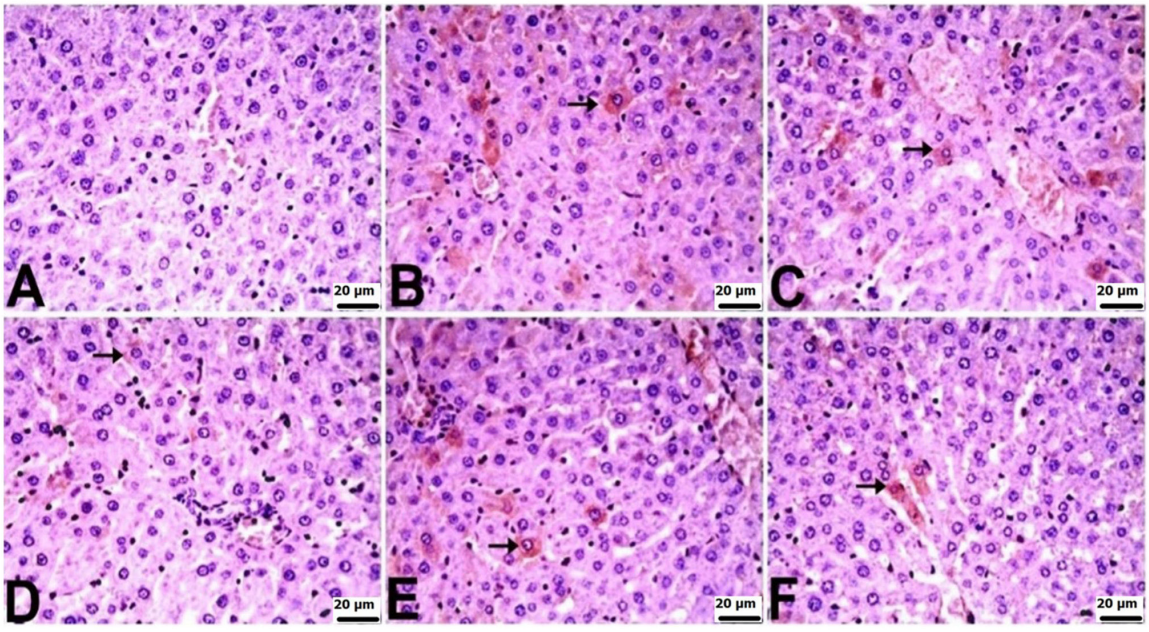

Figure 5. Immunohistochemical staining of interleukin-10 in the liver tissues per group (n=10). (A) Control Group: Immunonegativity. (B) CLP group: Middle level immunopositivity. (C) CLP + BX1 group: Middle level immunopositivity. (D) CLP + BX2 group: Light immunopositivity. (E) CLP + BA1 group: Middle level immunopositivity. (F) CLP + BA2 group: Light immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

The expression levels of miR-21 in the kidney tissue across the control, CLP and treatment groups are shown in Fig. 10A. While miR-21 expression levels were observed at baseline in the control group, a statistically significant decrease was detected in the CLP group compared with those in the control group. Similarly, in the CLP + BA1 and CLP + BX1 treatment groups, miR-21 levels remained lower than those in the control group, indicating that these treatments failed to restore miR-21 expression to baseline levels. By contrast, the CLP + BA2 group exhibited a statistically significant increase in miR-21 expression compared with that in the CLP group. The CLP + BX2 group demonstrated a moderate increase in miR-21 expression compared with that in the CLP group; however, this change was not statistically significant.

The mRNA expression levels of PTEN in the kidney tissue across the control, CLP and treatment groups are shown in

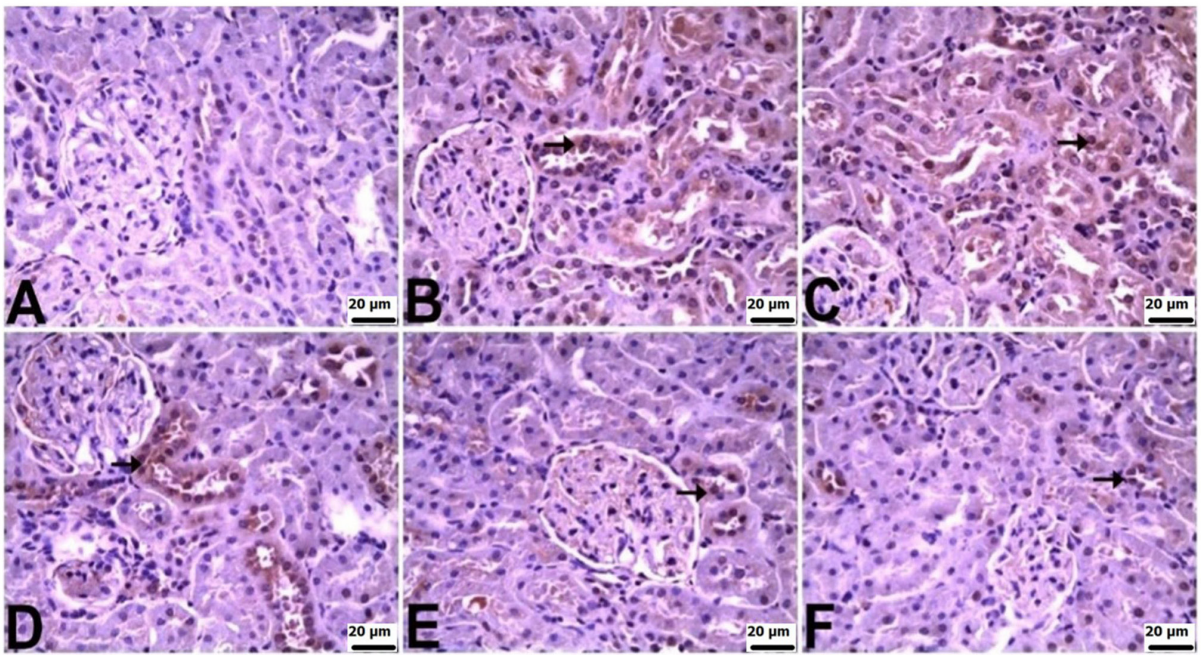

Figure 6. Immunohistochemical staining of tumor necrosis factor-α in the kidney tissues per group (n=10). (A) Control Group: Immunonegativity. (B) CLP group: Severe immunopositivity. (C) CLP + BX1 group: Moderate immunopositivity. (D) CLP + BX2 group: Moderate immunopositivity. (E) CLP + BA1 group: Light immunopositivity. (F) CLP + BA2 group: Light immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

Fig. 10B. In the control group, PTEN expression levels were observed to be the highest. In the CLP group, a significant decrease in PTEN expression was observed compared with that in the control group, suggesting that CLP-induced inflammation suppressed PTEN expression in kidney tissue. The CLP + BA1 group exhibited a significant increase in PTEN expression compared with that in the CLP group, indicating a potential restorative effect of BA1 treatment. However, in the CLP + BX1 and CLP + BX2 groups, PTEN expression levels remained significantly lower compared with those in the control group, suggesting that these treatments were unable to restore PTEN expression to baseline levels.

The mRNA expression levels of AKT in the kidney tissue across the control, CLP and treatment groups are shown in Fig. 10C. In the CLP group, a significant increase in AKT expression was observed compared with that in the control group; by contrast, the CLP + BA1, CLP + BA2, CLP + BX1 and CLP + BX2 groups showed a significant reduction in AKT expression compared with that in the CLP group, indicating that these treatments effectively suppressed AKT activation.

Table V. Statistical examination of immunopositivity in immunohistochemical staining conducted with TNF-α, IL-6 and IL-10 in the kidney tissues.

Groups	TNF-α	IL-6	IL-10
Control	0 (0.25)	0 (1.00)	0 (0.00)
CLP	3 (0.25)[a]	4 (0.25)[a]	2 (0.00)[a]
CLP + BX1	3 (0.25)[a]	3 (0.00)[b]	1 (0.00)[b]
CLP + BX2	2 (0.00)[b]	3 (0.00)[b]	1 (0.00)[b]
CLP + BA1	1 (0.00)[c]	1 (0.25)[c]	1 (0.00)[b]
CLP + BA2	1 (0.00)[c]	1 (0.00)[c]	1 (0.25)[b]

Values are represented as the median (IQR). [a-c]Signifies the variation among the groups ($P<0.05$). While the groups indicated with the same letter do not show statistical significance with each other, there is statistical significance at the level of $P<0.05$ between the groups with different letters. CLP, cecal ligation and puncture; BA, boric acid; BX, borax; TNF-α, tumor necrosis factor-α; IL, interleukin.

Discussion

Sepsis is a complex clinical manifestation characterized by insufficient oxygen delivery to tissues, driven by processes that induce microcirculatory changes (38). In the early phases, pro-inflammatory cytokines are released, initiating a robust inflammatory reaction leading to microcirculatory modifications. This disturbance affects cellular components, including endothelial cells, vascular smooth muscle cells, erythrocytes, leukocytes, platelets and parenchymal cells. Leukocyte activation amplifies inflammation, triggering platelet activation, the coagulation cascade and the complement system (39,40).

Cytokines, essential for cellular communication, are small proteins synthesized by macro-phages and helper T lymphocytes; they exert their effects not only on the secreting cells but also through autocrine, paracrine and endocrine actions. Typically released in a cascading manner, the stimulation of target cells by one cytokine triggers the production of others in a sequential cascade (41). In sepsis, they contribute to immunopathological processes by increasing the release of key inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and monocytic chemotactic protein (42). The resulting cytokine storm poses a potential threat, as it establishes a feedback loop between cytokines and immune cells. While the immune

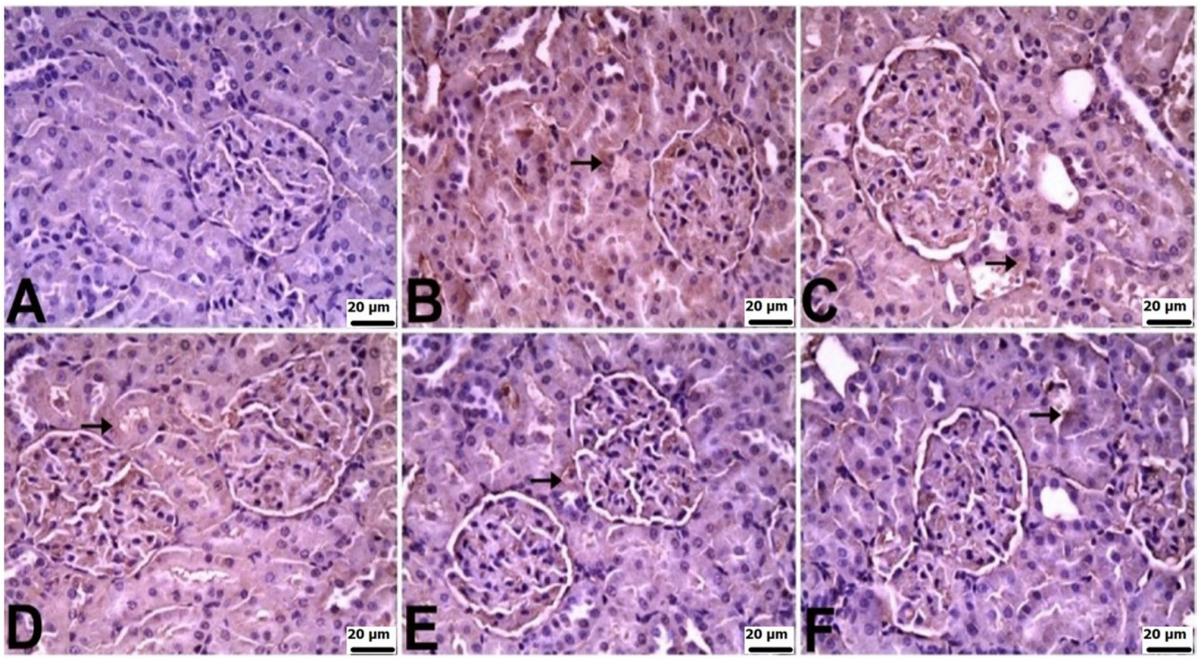

Figure 7. Immunohistochemical staining of interleukin-6 in the kidney tissues per group (n=10). (A) Control group: Immunonegativity. (B) CLP group: Very severe immunopositivity. (C) CLP + BX1 group: Very severe immunopositivity. (D) CLP + BX2 group: Very severe immunopositivity. (E) CLP + BA1 group: Light immunopositivity. (F) CLP + BA2 group: Light immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

system combats the pathogen, cytokines prompt lymphocytes and macrophages to migrate toward the inflamed site, encouraging the production of effector cytokines (43). The resulting cytokine storm establishes a feedback loop between cytokines and immune cells, potentially spiraling out of control, causing localized hyperactivation of immune cells and damage to inflamed organs. Circulating cytokines can also induce damage to distant organs (44).

TNF-α, primarily produced by activated macrophages but also synthesized by various cell types, (e.g., lymphocytes, neutrophils, mast cells and eosinophils), serves as an acute phase reactant and a signaling protein contributing to systemic inflammation; its main function involves the regulation of immune cells. IL-6, classified as a crucial mediator in the inflammatory response alongside other pro-inflammatory cytokines, such as IL-1 and TNF-α, is predominantly synthesized by fibroblasts, endothelial cells and monocytes (45). Originally termed a 'cytokine synthesis inhibitory factor', IL-10 functions by inhibiting the gene expression and synthesis of proinflammatory cytokines in macrophages, as well as T cell cytokines. Additionally, IL-10 suppresses the function of antigen-presenting cells (46).

Research data suggests that boron compounds (e.g., BA) could influence the synthesis of cytokines responsible for regulating reactive oxygen species (ROS) (47). Furthermore, BA is proposed to exhibit antioxidant characteristics by neutralizing protons on oxidative molecules. While BA actively participates in anti-inflammatory mechanisms, its specific advantages in the context of sepsis remain to be fully elucidated (48). Boron compounds play a role in modulating cell membrane functions, influencing transmembrane signaling and regulating the movement of ions. Additionally, they may serve as metabolic regulators in certain enzymatic systems. By elevating the levels of reduced glutathione, boron compounds inhibit the generation of ROS and apoptosis, thereby mitigating the impact of oxidative damage in the body (49,50). Research has demonstrated the hepatoprotective effects of boron compounds in countering carbon tetrachloride-induced liver degeneration (51). Another study underscored the protective capabilities of these compounds against damage induced by cyclophosphamide (52). In the present study, within the liver tissue, the CLP + BA2 group exhibited reduced expression levels of TNF-α and IL-6, classified as pro-inflammatory cytokines, whereas the level of IL-10, an anti-inflammatory cytokine, demonstrated a noteworthy increase in comparison with the control group. The histopathological evaluation of the liver in this particular group revealed lower score points indicative of necrosis and mononuclear cell infiltration compared with the other treatment groups. Similarly for BA administration, but for the kidney analysis, both the CLP + BA1 and CLP + BA2 groups exhibited a reduction in TNF-α and IL-6 levels, along with an increase in IL-10 levels. The histopathological assessment of these groups indicated a lower score for interstitial nephritis compared with that in the other treatment groups. The synthesis of diverse miRNAs, triggered by inflammation, governs the expression of molecules responsible for regulating the inflammatory process. Notably, miR-21, a highly expressed miRNA in numerous mammalian cells, orchestrates anti-inflammatory responses (53). Research indicates that miR-21 plays a crucial role in resolving inflammation by engaging in the negative feedback mechanism within inflammatory pathways. It has been proposed that overexpression of miR-21 in macro-phages leads to a reduction in TNF-α and IL-6 secretion, coupled with an increase in IL-10 production, underscoring the multifaceted nature of miR-21 in immune modulation (54). These findings also highlight

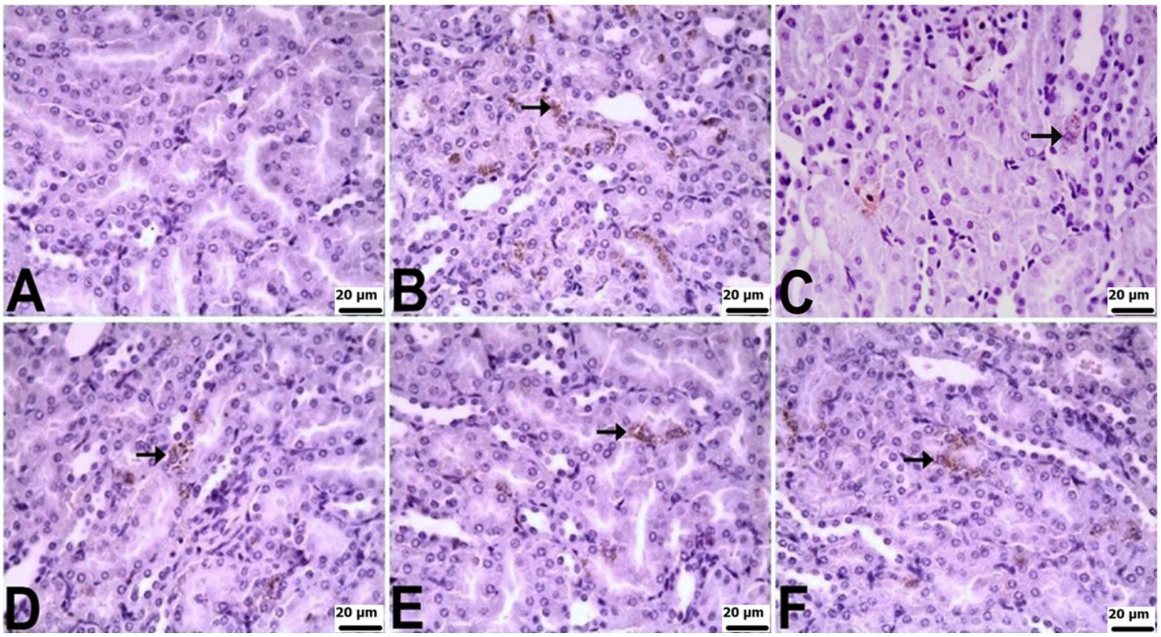

Figure 8. Immunohistochemical staining of interleukin-10 in the kidney tissues per group (n=10). (A) Control group: Immunonegativity. (B) CLP group: Moderate immunopositivity. (C) CLP + BX1 group: Light immunopositivity. (D) CLP + BX2 group: Light immunopositivity. (E) CLP + BA1 group: Light immunopositivity. (F) CLP + BA2 group: Light immunopositivity. Immunopositivity is indicated by the arrows. CLP, cecal ligation and puncture; BA, boric acid; BX, borax.

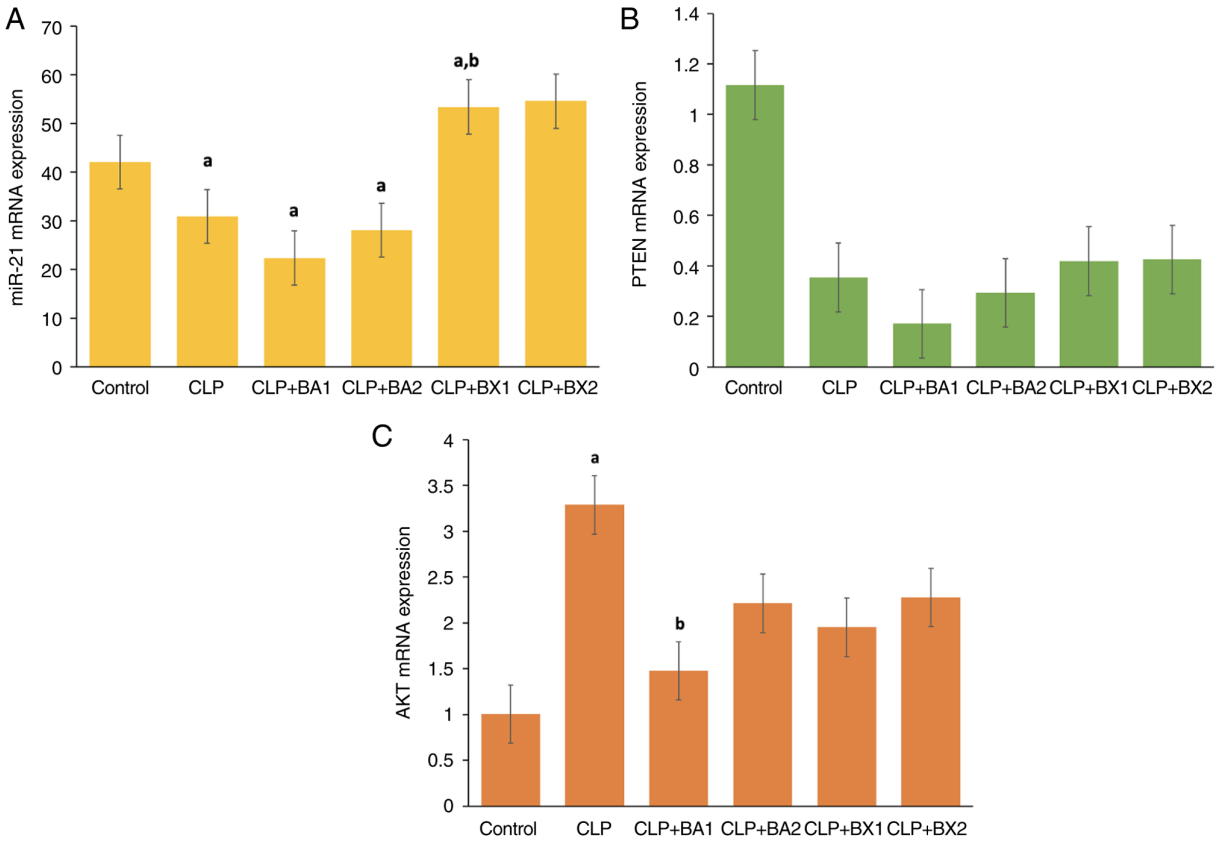

Figure 9. Gene expression levels in liver samples (n=10). (A) miR-21In, (B) PTEN and (C) AKT expression. [a]P<0.05 compared with the control group; [b]P<0.05 compared with the CLP group. CLP, cecal ligation and puncture; BA, boric acid; BX, borax; miR-21, microRNA-21; PTEN, phosphatase and tensin homolog.

the anti-inflammatory activity of miR-21, demonstrating its impact on the toll-like receptor 4-nuclear factor-κB (NF-κB) pathway and subsequent reduction in lipopolysaccharide (LPS)-induced inflammatory responses in macrophages. Due to its association with inflammation suppression and organ protection, miR-21 holds potential as a predictive marker for a

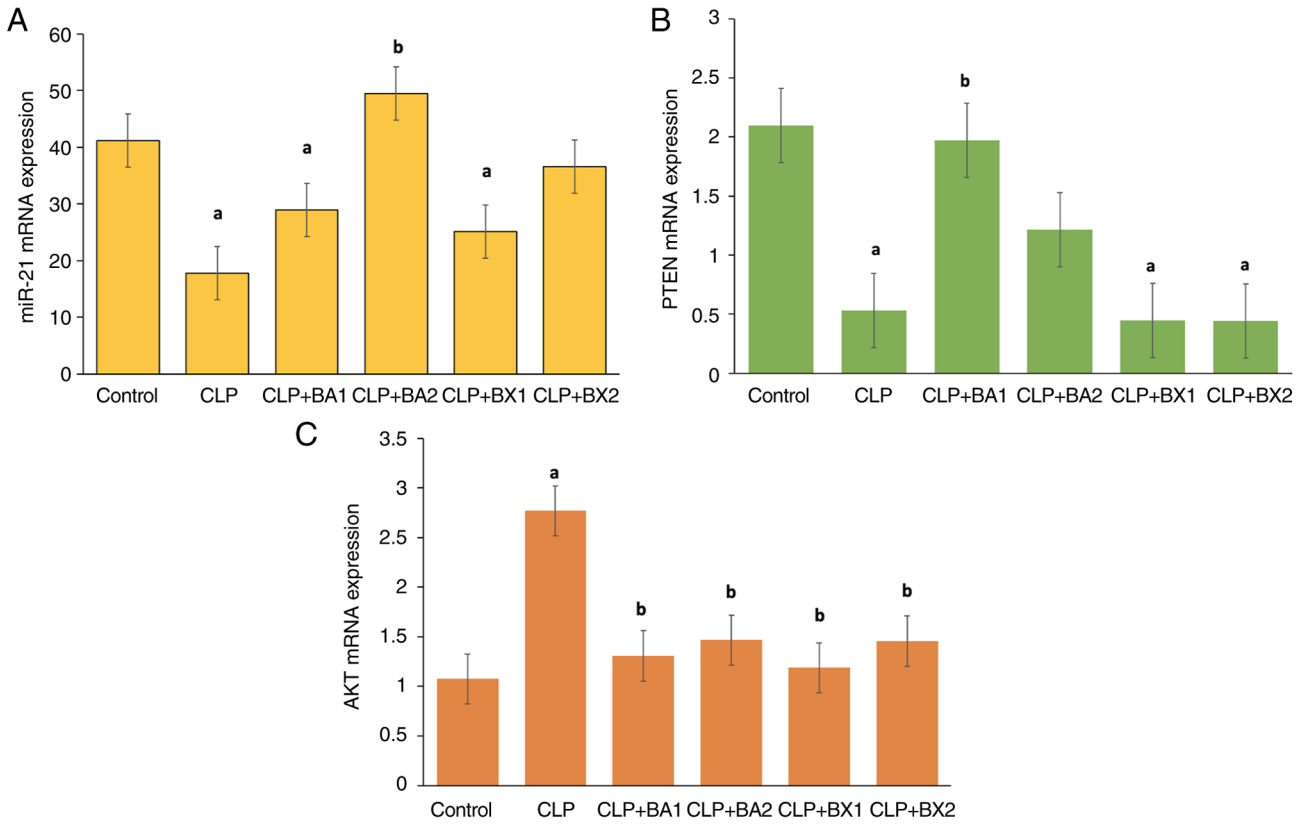

Figure 10. Gene expression levels in kidney samples (n=10). (A) miR-21, (B) PTEN and (C) AKT expression. [a]P<0.05 compared with control group; [b]P<0.05 compared with CLP group. CLP, cecal ligation and puncture; BA, boric acid; BX, borax; miR-21, microRNA-21; PTEN, phosphatase and tensin homolog.

decreased risk of sepsis (54). In the present study, the miR-21 levels were significantly increased in the liver tissues of the CLP + BX1 and CLP + BX2 groups (not significant), and in the kidney tissues of the CLP + BA2 group, compared with the control group. The marked increase of miR-21 levels in the liver for the CLP + BX1 and CLP + BX2 groups, relative to the CLP group, was associated with decreased TNF-α and IL-6 levels, along with increased IL-10 levels. Additionally, the CLP + BA2 group exhibited similar effects in the kidney tissue.

The PTEN gene, recognized as phosphatase and tensin homolog deleted on chromosome 10, interferes with diverse cellular processes, such as cellular growth, proliferation and movement, by counteracting PI3K. Further comprehensive exploration is required to understand the means through which PTEN expression is controlled (55). Research conducted by Kim *et al* (56) revealed that exposing cancer cell lines to TNF-α results in the reduction of PTEN expression, while TNF-α/NF-κB promotes the conveyance of the TNF-α signal, leading to the activation of AKT. The down-regulation of PTEN by NF-κB underscores its involvement in regulating PI3K/AKT, given that TNF-α/TNF receptor also conveys signals through the activation of the PI3K/AKT pathway (56). Studies have demonstrated heightened baseline levels of AKT phosphorylation in the tumors of mice lacking PTEN. The present study findings demonstrated that PTEN expression was markedly diminished in the liver and kidney tissues in the CLP group. However, notably, it was observed that the PTEN expression within the kidney tissue of the CLP + BA1 group was at a similar level compared with that of the control group.

PI3K, where the primary downstream kinase is AKT, constitutes a widely distributed family of protein kinases engaged in signal transduction. This intricate process is mediated through receptor tyrosine kinases or G protein-coupled receptors, which include TNF-α receptors (57). In macrophages, the activation of PI3K responds to LPS, setting off a series of coordinated events that consecutively activate AKT, resulting in its phosphorylation. This phosphorylation, in a cascading fashion, triggers the activation of various downstream targets, orchestrating diverse cellular functions such as nuclear factor-κB or PI3K/AKT pathways (58,59). In the present study, within the kidney tissues, the CLP group exhibited elevated AKT gene expression levels in comparison with the control group. However, there was no alteration observed in the treatment groups. In the liver tissues, both the CLP and treatment groups displayed a notable rise in AKT levels compared with the control group. Nevertheless, within the treatment groups, AKT levels experienced a substantial reduction in comparison with those in the CLP group. Within these treatment groups, there was a notable reduction in TNF-α and IL-6 levels compared with the CLP group, accompanied by an increase in IL-10 levels.

In the present study, the primary emphasis was on assessing the impact of the natural compounds BA and BX on pro-inflammatory and anti-inflammatory parameters within the liver and kidney tissues, recognized as target organs in the CLP-induced sepsis model. It is worth mentioning that despite the accumulated evidence as to their mechanisms of action and despite being present in >400 different products, such as fertilizers, pesticides, cosmetics, medicines, food supplements,

cleaning agents and personal care items, boron compounds have not undergone widespread clinical evaluation due to misconceptions about potential serious toxic effects. Recent research has debunked these concerns, revealing that boron compounds do not induce toxicity in healthy cells (28,29,60). The present results further support the notion that boron compounds could be further explored with regard to their pharmacological actions against inflammatory processes.

As our understanding of the anti-inflammatory properties of these compounds is limited, it becomes essential to unveil the mechanisms underlying their anti-inflammatory activity. In the present investigation, in liver tissue, the CLP + BX1 and CLP + BX2 groups exhibited elevated miR-21 expression; whereas in kidney tissue, only the CLP + BA2 group exhibited elevated miR-21 expression. Additionally, these groups showed the downregulation of PTEN expression and the upregulation of AKT expression. The findings led to the conclusion that, despite a decrease in TNF-α and IL-6 levels, there was a notable increase in miR-21 levels within this group when compared with those in the sepsis group. While these results align with each other, more intricate analyses are required to elucidate the mechanism in greater detail.

The present study has several limitations that should be acknowledged. Firstly, while the use of boron compounds, such as BA and BX has shown promising anti-inflammatory effects in sepsis-induced liver and kidney damage in a rat model, these findings are limited to preclinical settings and may not directly translate to clinical applications. Secondly, the present study focused on specific pathways and biomarkers, such as miR-21/PTEN/AKT, which does not encompass the full spectrum of potential inflammatory and molecular interactions that may occur in sepsis. Furthermore, the relatively short experimental duration of 24 h post-sepsis induction restricts the evaluation of long-term effects and potential recovery outcomes. Finally, although efforts were made to standardize treatment dosages and conditions, individual variability among the animal models could not be entirely accounted for. Future research should include a broader range of experimental conditions, prolonged observation periods, and clinical trials to validate these findings and explore the detailed mechanisms of boron compounds in mitigating sepsis-related organ damage. Despite the limitations, the present investigation underscores the necessity for further research into the potential benefits of using a low dose of BA to alleviate kidney damage caused by sepsis.

Given that the early release of pro-inflammatory cytokines during sepsis initiates a robust inflammatory response, leading to microcirculation alterations, the prompt organization and management of this cytokine storm are imperative. Boron compounds have recently gained popularity across various fields. The present study demonstrates the notable anti-inflammatory effects of boron compounds in rats with induced sepsis. BA, in particular, showcased promising results in mitigating kidney damage. Immunohistochemical analyses revealed a significant reduction in pro-inflammatory markers (TNF-α and IL-6) and an increase in the anti-inflammatory marker IL-10. These findings suggest the potential of BA as a potential new agent to alleviate sepsis-induced inflammatory responses, proposing the need for further research. The present study underscores the need for detailed investigations into the underlying mechanisms and supports exploring the use of low-dose BA and BX for managing sepsis-induced organ damage. Overall, the results contribute valuable insights to the understanding of the pharmacological actions of boron compounds in inflammatory conditions.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CS and ATs conceptualized and designed the study. MO, MK, SG, ASM and YY engaged in the acquisition, analysis and interpretation of the data. ATa, CS, MS and SVK contributed to the interpretation of the data, along with manuscript drafting and finalization. MS, ATa, EO and DAS were involved in the interpretation of the data and in critical revisions of the intellectual content. CS, DAS and ATs confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study complied with the ethical guidelines approved by the ethical committee, and all procedures involving animal research were conducted in accordance with Directives 2010/63/EU, which regulate the protection and welfare of animals used for scientific purposes within the European Union. Approval for these procedures was granted by the Ethics Committee at Kastamonu University (approval no. 28/4). The study also complied with ARRIVE guidelines and the AVMA euthanasia guidelines 2020.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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