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Prophylactic and Therapeutic Hydroxychloroquine and Zinc Administration Do Not Exert Synergistic Renal Protection in a Rat Sepsis Model

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Abstract

Purpose

The aim of this study was to evaluate whether combined administration of hydroxychloroquine and zinc cause more damage or synergistic protection against sepsis-induced renal injury in a cecal ligation and puncture (CLP) rat model.

Methods

A total of Sprague–Dawley rat model 30 rats were used in the study, divided into five groups of six rats each: Sepsis (SE), Zinc + Sepsis (Z), HCQ + Sepsis (H), and HCQ + Zinc + Sepsis (HC) and Sham (Sh). The groups were treated with zinc, hydroxychloroquine, combined treatment or no treatment for 8 days and sepsis model was induced via cecal ligation and puncture (CLP) in 6th day.

Results

Histopathological renal injury scores for Z, H and HC groups were significantly lower compared to SE group ($p < 0.001$). However, no statistically significant differences were observed among Z, H and HC group. White blood cell (WBC) levels were significantly higher in SE group compared to Z, H, and Sh group ($p < 0.05$). No significant differences in WBC levels were observed among Z, H and HC and Sh group. Neutrophil analysis revealed no significant differences between Z, HC and SE groups but HC group had significantly higher neutrophil levels compared to Sh group ($p < 0.05$). Additionally, no significant differences in neutrophil levels were found among Groups SE, Z, H and Sh group.

Conclusion

Despite the known zinc ionophore activity of hydroxychloroquine, combined hydroxychloroquine and zinc treatment did not provide additional renal protection compared to monotherapy.

1. Introduction

Hydroxychloroquine was first described as an antimalarial drug, but subsequently has been used in the treatment of rheumatic diseases that increase the secretion of proinflammatory cytokines [1]. Zinc is an essential trace element and functions as a co-factor, especially for the cells of the defense system [2]. If zinc is deficient in the body, it can cause complaints such as growth retardation, dermatitis or prolonged wound healing time, lymphopenia or low resistance to infection [3]. When zinc is administered to the body, the increase in the zinc level occurring in the cell is lower than the amount of zinc administered. The structure of the cell membrane prevents the entry of zinc into the cell. When hydroxychloroquine is administered to the body, it has been observed that zinc causes a significant increase in the intracellular level, which supports that hydroxychloroquine is a zinc ionophore [4]. While previous studies have demonstrated the individual effects of zinc and hydroxychloroquine on the immune system in sepsis, it remains unclear whether their combined administration enhances each other's effects. Kidney is one of the most affected organs from inflammation and acute kidney injury has high mortality during sepsis [5]. The effects of hydroxychloroquine and zinc have been shown to reduce organ damage independently of each other in sepsis, however, it remains unclear whether the increased intracellular zinc levels mediated by hydroxychloroquine translate into additive or synergistic sepsis related acute kidney injury protection.

1.1 Sepsis

Sepsis is a systemic response of the body against microbial pathogens and causes morbidity and mortality by causing damage to its own tissues and organs. In case of severe sepsis, early diagnosis, rapid initiation of antibiotics and early aggressive resuscitation are the most important interventions in the emergency department [4].

1.2 Zinc

Zinc is an element used by almost all immune cells [6]. For example, immature T cells require zinc to differentiate. Furthermore, zinc has been shown to facilitate the development of Treg cells and inhibit some T cell mediated diseases such as autoimmune diseases, allergy and transplantation by stabilazing Treg cells [7]. At the molecular level, zinc also has a second messenger role in immune cells. Decrease or increase in intracellular free zinc concentration is also a "zinc signal". Zinc signalling is involved in events such as the generation of

proinflammatory cytokines by monocytes, the presentation of majör histocompatibility complex (MHC) class II molecules on the major of dendritic cells, the formation of neutrophil traps by neutrophil granulocytes or the increase in the number of T cells [8].

1.3 Hydroxychloroquine

Hydroxychloroquine can inhibit immune system activation, antigen presentation and MHC II expression in immune system cells, especially B and T cells, by using the Toll-like receptor [9]. Some immunomodulatory mechanisms of hydroxychloroquine are inhibition of cytokine production, decrease in chemokine levels, inhibition of microRNA expression, decrease in TH17-related cytokines, decrease IFN-α, IL-2, IL-10 levels, cytotoxic T cell inhibition activities [10]. They can also reduce the production of immune system proinflammatory cytokines such as IL-1, IL-6, IFN-γ and TNF-α [11].

The aim of our study is to demonstrate the effect of increased intracellular zinc levels following hydroxychloroquine treatment in the context of sepsis on organ damage.

2. MATERIALS AND METHODS

This study protocol has been approved by the Acıbadem Mehmet Ali Aydınlar University Local Animal Experiments Ethics Committee (Approval No: 2021/32, Decision Date: 18.05.2021). Animal experiments were conducted at the AAALAC accredited Acibadem Mehmet Ali Aydınlar University, Experimental Animals Application and Research Center. Throughout this study, ethical principles outlined in the Acıbadem Mehmet Ali Aydınlar University Local Animal Experiments Ethics Committee Guidelines were adhered too. Financial support for the study was provided by the Istanbul Medeniyet University Scientific Research Projects Unit with Project ID:1737.

Clinical Trial Number

Not applicable.

2.1 Experimental Animals

A total of 43 Sprague-Dawley rats, each weighing between 250 and 300 grams, were used for the study. Rats were fed with standard pellet rat chow containing 130 mg/kg zinc in a light-controlled (12 hours of light and 12 hours of darkness) environment with temperature (22 ± 1˚C) and humidity (65–70%) [12].

Table 1
Animal Numbers

Group 1	**Sepsis**	**9**
Group 2	Sepsis + Zinc (40 mg/kg)	9
Group 3	Sepsis + Hydroxychloroquine (200mg/kg)	9
Group 4	Zinc (40 mg/kg) +Hydroxychloroquine (200 mg/kg)	9
Group 5	Sham (Control)	7
Total		43

Although 43 rats were initially enrolled, CLP model releated mortality resulted in six animals per group being included in the final analysis (Table 1).

2.2 Experiment Protocol

To prepare the sepsis model, 50 mcg/kg acetaminophen was administered intraperitoneally 15 min before the procedure. Under general anesthesia (Xylazine 3 mg/kg and ketamine 100 mg/kg intraperitoneally), an incision was made in the mid-upper abdomen of the rats including the peritoneum and the cecum was reached. 2 – 0 silk sutures have been used to ligate the cecum from 1 cm beyond of the ileocecal junction and punctured at two points using a 21-gauge needle to make a severe sepsis model. After this procedure, pressure was applied to the cecum to spread the fecal contents around the cecum and contaminate the abdominal cavity. The cecum was then returned to its original anatomical position and the abdominal incision was sutured. Then, 30cc/kg of fluid was injected subcutaneously to compensate for the fluid lost during surgery. In the sham group, after the cecum was reached, the abdomen was closed without perforation to return the cecum to its anatomical position [12].

2.3 EXPERIMENTAL GROUPS

Group 1 (Sepsis, n = 9): Sepsis was induced on the 6th day in 9 rats, which were provided with standard pellet food and water for 5 days.

Group 2 (Sepsis+Zinc, n = 9): Sepsis was induced on the 6th day in 9 rats, which were provided with standard pellet food and water for 5 days. Additionally, 40 mg/kg Zinc Chloride was administered via orogastric tube every 12 hours in the morning and evening. The last treatment was given on the evening of the 7th day.

Group 3 (Sepsis+Hydroxychloroquine, n = 9): Sepsis was induced on the 6th day in 9 rats, which were provided with standard pellet food and water for 5 days. Additionally, 200 mg/kg of hydroxychloroquine was administered via orogastric tube every 12 hours in the morning and evening. The last treatment was given on the evening of the 7th day.

Group 4 (Sepsis+Hydroxychloroquine+Zinc, n = 9): Sepsis was induced on the 6th day in 9 rats, which were provided with standard pellet food and water for 5 days. Additionally, 40 mg/kg of zinc chloride and 200 mg/kg of hydroxychloroquine were administered via orogastric tube every 12 hours in the morning and evening. The last treatment was given on the evening of the 7th day.

Group 5 (Sham, n = 7): Seven rats were provided with standard pellet food and water for 5 days. On the 6th day, under general anesthesia, an incision was made at the mid-upper line of the rats' abdomen, reaching the cecum. The cecum was sutured at the midline of the abdomen without ligation or perforation, and the abdomen was closed.

All rats were sacrificed on the 8th day under anesthesia (Ketamine 100 mg/kg and Xylazine 3 mg/kg intraoperitoneal) via cardiac puncture. Animals that died before the sacrifice (1 from the sepsis group, 1 from the hydroxychloroquine group, 1 from the zinc group, and 1 from the zinc+hydroxychloroquine group) were excluded from the study.

2.4 Collection of Samples

At the end of the experimental period, sacrification has been done via cardiac puncture under general anesthesia and during sacrification 8–10 cc blood was collected [12]. The collected blood was processed at the Istanbul Medeniyet University Faculty of Medicine Biochemistry Laboratory using commercial kits with hemogram tube (purple-colored) and SST gel tube (yellow-capped) in containers refrigerated at + 4 degrees Celsius. CRP, IL-6, procalcitonin and hemogram levels were measured in blood samples obtained from rats. Kidney, heart, lung, spleen, and liver tissues were collected for histological examination. For 3 days, these tissue samples were fixed in 10% neutral buffered formalin solution. After fixation, the tissues underwent dehydration using an ascending series of ethyl alcohol concentrations (70%, 90%, 96%, 100%) and were then cleared with xylene (Fig. 1).

Subsequently, the tissues were embedded in paraffin. Sections, cut at a thickness of 5 µm using a microtome, were transferred to a warm water bath. The sections obtained from tissue blocks were then placed on slides for histological staining.

3. HISTOLOGICAL EVALUATION

3.1 Tissue processing

The tissues fixed in a 10% neutral formalin solution has been washed with tap water for 5 hours. They were then soaked in 70% alcohol overnight. The following day, they were sequentially passed through 1 hour of 80% alcohol, 1 hour of 90% alcohol, two periods of 30 minutes each in 96% alcohol, and two periods of 30 minutes each in 100% alcohol. It was incubated in xylene for two periods of 15 minutes each. Subsequently, it was immersed in a mixture of melted paraffin and xylene for 45 minutes. Finally, it was kept in paraffin wax for 2 hours. Afterwards, the tissues were embedded in paraffin blocks. The tissues were then stained with Periodic Acid-Schiff (PAS) and Hematoxylin-Eosin (H&E) [12].

Parameters examined:

3.2.1 Glomerular area;

Increase in Bowman's space: Volumetric expansion in the Bowman's space of glomeruli in the renal cortex has been investigated [13].

Glomerular atrophy: Volumetric decrease in the capillary tuft accompanied by volumetric expansion in the Bowman's space of kidney glomeruli has been examined [14].

Vacuolization in the glomerulus: The appearance of vacuoles indicating capillary necrosis in kidney glomeruli has been assessed [14].

Cells in the glomerular area have been counted, ranging from 0 to 40.

3.2.2 Tubular area

Table 2
Scoring used for tubular parameters.

Cell number observed in the section.	Score
1 or 2	Few
3 or 4	Moderate
5 and more	Many

Tubular dilation: An increase in volume has been sought in renal tubules [14]. Tubulopitelial necrosis: With dilation in the renal tubules, vacuolization has been investigated (Table 2).

Cast formation: Formation of casts with protein precipitation in the renal tubules has been investigated (Table 2) [15].

3.2.3 Interstitial area;

Interstitial hemorrhage: The presence of erythrocytes in the renal interstitium has been investigated [16].

Mononuclear infiltration: The presence of lymphocytes and monocytes in the renal interstitium has been investigated [16].

Sections that have been stained were scored based on the degree of tissue damage, including tubular and glomerular degeneration, inflammatory cell infiltration, and the degree of congestion on a scale of 0 to 5 (0: no damage; 1: minimal damage; 2-3-4: moderate damage; 5: severe damage) (Table 2).

Histopathological evaluation was performed by a pathologist blinded to the experimental groups.

4. STATISTICAL EVALUATION

The SPSS (Statistical Package for Social Sciences) software package (for Windows; version 18.0) (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the obtained data. Data distribution was assessed using the Shapiro–Wilk test. As the data were not normally distributed, comparisons among groups were performed using the Kruskal–Wallis test, followed by Conover post hoc analysis. A p value $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) was considered statistically significant [17].

5. RESULTS

5.1 Microscopic Findings

Table 3
Histopathological statistics

Groups	n	Tubular Dilatation ($\bar{x} \pm S\,\bar{x}$)	TuboEpitelial Necrosis ($\bar{x} \pm S\,\bar{x}$)	Cast Formation ($\bar{x} \pm S\,\bar{x}$)	Glomerular Vacuolization ($\bar{x} \pm S\,\bar{x}$)	Bowman Space Dilatation ($\bar{x} \pm S\,\bar{x}$)	Glomerular Atrophy ($\bar{x} \pm S\,\bar{x}$)	Mononuclear Infiltration ($\bar{x} \pm S\,\bar{x}$)	Hemorrhagea ($\bar{x} \pm S\,\bar{x}$)
SH	6	2.25 ± 0.25^{c}	0.17 ± 0.11^{b}	0.25 ± 0.13^{bc}	0.83 ± 0.27^{b}	3.92 ± 0.48^{ac}	0.50 ± 0.19^{b}	0.25 ± 0.13^{b}	1.33 ± 0.41^{bc}
SE	6	7.83 ± 0.84^{a}	3.25 ± 0.41^{a}	1.38 ± 0.32^{a}	3.50 ± 0.46^{a}	5.92 ± 0.57^{a}	2.92 ± 0.60^{a}	2.88 ± 0.40^{a}	4.75 ± 0.90^{a}
H	6	2.92 ± 0.63^{bc}	0.91 ± 0.39^{bc}	0.38 ± 0.18^{ac}	0.90 ± 0.23^{b}	2.81 ± 0.35^{bc}	1.10 ± 0.31^{ab}	1.00 ± 0.33^{b}	3.25 ± 0.53^{ac}
Z	6	3.25 ± 0.41^{bc}	0.58 ± 0.29^{bd}	0.00 ± 0.00^{c}	2.57 ± 0.43^{a}	1.75 ± 0.49^{b}	0.42 ± 0.19^{b}	1.14 ± 0.40^{b}	2.86 ± 0.74^{ac}
HC	6	3.67 ± 0.36^{b}	1.67 ± 0.38^{acd}	0.17 ± 0.17^{c}	4.83 ± 0.60^{a}	2.33 ± 0.36^{bc}	0.75 ± 0.18^{b}	1.00 ± 0.45^{b}	0.00 ± 0.00^{b}
p		***	***	**	***	***	**	**	***

Values are presented as mean +/- standard error of the mean ($\bar{x} \pm S\bar{x}$). Groups: SH: Sham, SE: Sepsis, H: Hydroxychloroquine, Z: Zinc, HC: Hydroxychloroquine+Zinc.

[a,b,c,d] Means within each grouping with different letter designations differ significantly.

**: $p < 0.01$, ***: $p < 0.001$, n: number of rats,

$\bar{x}$: mean, $S\bar{x}$: Standard error.

The morphological images of the sections belonging to the control group were observed to be within normal histological structure. In the sepsis group, severe glomerular and tubular damage were observed.

For Bowman's space dilation (Fig. 2), the sepsis group was only numerically higher than the control group ($p > 0.05$), with no significant difference between them. However, the hydroxychloroquine, zinc, and combined zinc and hydroxychloroquine groups were significantly lower than the sepsis group.

In terms of glomerular atrophy (Fig. 2), no difference was observed among the control, hydroxychloroquine, zinc, and combined zinc and hydroxychloroquine groups. These groups were significantly lower than the sepsis group. The hydroxychloroquine group had numerically fewer cases than the sepsis group, but the difference was not statistically significant (Table 3).

a: This is a kidney glomerulus stained with H&E in the control group. The star symbol represents a healthy glomerulus, while the arrowhead indicates the Bowman's capsule. b: This is a kidney glomerulus stained with H&E in the sepsis group. The star symbol indicates glomerular atrophy, and the arrowhead shows Bowman's capsule enlargement c: This is a kidney glomerulus stained with H&E in the Zinc group. The arrowhead indicates Bowman's capsule enlargement, and the arrow symbol represents glomerular vacuolization. d: This is a kidney glomerulus stained with H&E in the Hydroxychloroquine group. The arrow symbol indicates glomerular vacuolization, while the star symbol represents glomerular atrophy. e: This is a kidney glomerulus stained with H&E in the group treated with Hydroxychloroquine along with zinc. The arrow symbols indicate vacuolization, while the arrowheads show Bowman's capsule enlargement.

Regarding hemorrhage (Fig. 3), the hydroxychloroquine, zinc, and combined zinc and hydroxychloroquine groups were similar to the control group. Among the treatment groups, only the group receiving both hydroxychloroquine and zinc was significantly lower than the sepsis group, as well as the hydroxychloroquine and zinc groups individually (Table 3).

a: Control group H&E-stained normal renal interstitial tissue b: The interstitium of the kidney tissue stained with H&E in the sepsis group shows arrowhead bleeding, indicating star sign mononuclear infiltration. c: Zinc group H&E-stained normal kidney interstitium. d: In the Hydroxychloroquine group, H&E stained interstitium reveals star sign bleeding. e: The interstitium stained with H&E in the group treated with Hydroxychloroquine and zinc together shows star sign bleeding and mononuclear infiltration.

Regarding cast formation (Fig. 4), there was no difference among the control, hydroxychloroquine, zinc, and the combined zinc and hydroxychloroquine groups. However, these groups were significantly lower than the sepsis group. The hydroxychloroquine group had numerically fewer cases than the sepsis group, but the difference was not statistically significant (Table 3).

In the examination of tubular epithelial necrosis (Fig. 4), no statistical difference was observed among the control, hydroxychloroquine, and zinc groups. These three groups had significantly less necrosis compared to the sepsis group. Although the group receiving both zinc and hydroxychloroquine showed fewer cases than the sepsis group numerically, the difference was not statistically significant.

In the examination conducted regarding tubular dilation (Fig. 4), it was found that the group receiving control, hydroxychloroquine, zinc, and zinc along with hydroxychloroquine had significantly lower values compared to the sepsis group ($p < 0.001$). However, no statistically significant difference was found between the group receiving hydroxychloroquine alone and the group receiving both hydroxychloroquine and zinc. And also, no significant differences were observed among the control, hydroxychloroquine, and zinc groups (Table 3).

a: Belonging to the control group, this is an H&E-stained kidney tissue, showing star sign healthy kidney tubules b: This is an H&E-stained kidney tubule belonging to the sepsis group, showing star sign cast formation, arrowhead tubular dilation, and necrosis. c: It is a kidney tubule belonging to the Zinc group, stained with H&E, showing star-shaped tubular dilation. d: Belonging to the tubule of hydroxychloroquine group which is stained H&E; star sign is dilated distal tubule arrowhead is dilated proxymal tubule e: This is a tubule of hydroxychloroquine+zinc group-stained H&E and star sign show tubuler dilatation

All treatment groups demonstrated significantly reduced renal injury compared to the sepsis group; however, combined hydroxychloroquine and zinc therapy did not show superiority over monotherapy (Table 3).

5.2 BIOCHEMICAL ASSESSMENT

In the analysis of WBC levels, the group receiving hydroxychloroquine combined with zinc in the sepsis-induced group showed a numerically higher value, though this difference was not statistically significant. The sepsis group, however, had significantly higher WBC levels compared to the hydroxychloroquine, zinc, and sham groups ($p < 0.05$). No significant differences were observed among the groups receiving hydroxychloroquine, zinc, hydroxychloroquine combined with zinc, and the sham group.

In the analysis of neutrophil counts, no significant difference was found between the group receiving zinc with hydroxychloroquine and the sepsis group. However, the zinc and hydroxychloroquine group had significantly higher neutrophil counts compared to the sham group ($p < 0.05$). No significant differences were observed among the hydroxychloroquine, zinc, sepsis, and sham groups (Table 4).

Table 4
Statistical Results of Biochemical Parameters.

Groups	**n**	**Wbc** ($\bar{x} \pm S_{\bar{x}}$)	**Neutrophils** ($\bar{x} \pm S_{\bar{x}}$)	**Lymphocytes** ($\bar{x} \pm S_{\bar{x}}$)
Group 1 Sepsis	6	6.73 ± 0.86^{a}	2.58 ± 1.18^{ab}	3.13 ± 0.62^{a}
Group 2 Sepsis+Zinc	6	3.93 ± 0.14^{c}	1.58 ± 0.13^{ab}	2.07 ± 0.17^{ab}
Group 3 Sepsis+ Hydroxychloroquine	6	3.67 ± 0.54^{c}	1.88 ± 0.55^{b}	1.40 ± 0.21^{b}
Group 4 Sepsis+Hydroxychloroquine+Zinc	6	4.58 ± 0.48^{ac}	3.42 ± 0.46^{a}	1.46 ± 0.42^{b}
Group 5 Sham-Control	6	4.20 ± 0.88^{c}	1.08 ± 0.25^{b}	2.70 ± 0.61^{ab}
P		* (0.023)	* (0.037)	* (0.039)

In the examination of IL-6 and PCT levels, no significant differences were observed in IL-6 and procalcitonin levels among any of the groups (Table 5).

Table 5
IL-6, PCT Results

Groups	**n**	**IL-6** ($\bar{x} \pm S_{\bar{x}}$)	**PCT** ($\bar{x} \pm S_{\bar{x}}$)
H	6	67.08 ± 3.59^{c}	1161.83 ± 76.88
Z	6	68.91 ± 3.18^{c}	1232.48 ± 77.41
HC	6	80.15 ± 4.07^{bc}	1184.08 ± 63.00
S	6	95.04 ± 5.85^{ab}	1314.82 ± 51.90
SH	6	102.40 ± 9.25^{a}	1392.93 ± 68.99
p		*** (0.000)	NS (0.130)

^{a,b,c} Means within each grouping with different letter designations differ significantly.

***: $p < 0.001$, n: number of rats, IL-6: Interleukin-6, PCT: Procalcitonin

$\bar{x}$: mean, $S_{\bar{x}}$: Standard error.

6. Discussion

Zinc and hydroxychloroquine have been histopathologically evaluated separately for kidney and the results were attenuated sepsis-related renal damage compared to untreated sepsis. Our study evaluated the effects of zinc, hydroxychloroquine, and their combined

administration. Their combined administration did not result in an additive or synergistic protective effect. These findings suggest that increasing intracellular zinc levels through hydroxychloroquine-mediated ionophore activity may not translate into enhanced organ protection in the context of experimental sepsis.

Zinc has been shown to stabilize cellular membranes, reduce oxidative stress, and modulate inflammatory signaling pathways, thereby limiting tubular and glomerular injury during systemic inflammation. In experimental sepsis models, zinc deficiency has been associated with exaggerated inflammatory responses and increased organ injury, while zinc repletion limits tissue damage and improves survival [2]. Similarly, hydroxychloroquine exerts anti-inflammatory effects by inhibiting Toll-like receptor signaling, antigen presentation, and proinflammatory cytokine production, which may contribute to reduced renal tissue damage in sepsis [5].

Sepsis causes acute kidney injury, and these destructive findings can be observed in glomerular, tubular, and immune system cells within the interstitial tissue of the kidney, histologically [18]. We focused on various parameters such as tubular dilation, cast formation, bowman's space dilation, glomerular atrophy, tubular epithelial necrosis and mononuclear lymphocyte infiltration in our study and sepsis group, the evaluations for the control group, hydroxychloroquine group, zinc group, and the group receiving both hydroxychloroquine and zinc together were significantly lower ($p < 0.001$). However, there was no statistically significant difference observed among the hydroxychloroquine group, zinc group, and the group receiving both hydroxychloroquine and zinc together.

Leukocytes, especially endothelial cells, peripheral blood mononuclear cells and macrophage-activated adipocytes can potentially contribute to the production of thyroid non-procalcitonin (ProCT) in vivo [19]. Furthermore, parenchymal cells in infected tissue cause a significant increase in circulating ProCT and NPCT (possibly non-procalcitonin) [20]. In our study, the examination of leukocytes revealed a numerically higher count in the group where sepsis was induced and zinc was given along with hydroxychloroquine, compared to the sepsis-only group. However, there were no statistically significant difference. The sepsis group was determined to be statistically higher than the hydroxychloroquine, zinc, and sham groups ($p < 0.05$). There was no significant difference between the hydroxychloroquine, zinc, hydroxychloroquine with zinc, and sham groups.

Neutrophils play a particularly important role in blocking bacterial dissemination in the body and are the first line of defense of the immune system; the movement of polymorphonuclear neutrophils (PMN) to the site of infection is absolutely essential in controlling the infection in sepsis [21]. However, excessive or prolonged neutrophil activation may exacerbate tissue injury through the release of proteases and reactive oxygen species [22]. In the examination of neutrophils, it was determined that there was no significant difference between the Zinc, hydroxychloroquine with zinc, and Sepsis groups. However, the group receiving hydroxychloroquine with zinc was found to be significantly higher than the Sham group ($p < 0.05$). Additionally, there was no difference between the Hydroxychloroquine, Zinc, Sepsis, and Sham groups.

Lymphocyte apoptosis and dysfunction are recognized as central features of sepsis-related immunosuppression. Previous studies have demonstrated that lymphocyte depletion contributes to impaired host defense in sepsis [23]. In the examination of lymphocytes, it was found that the Sepsis group was significantly higher than the Hydroxychloroquine and Hydroxychloroquine + Zinc groups ($p < 0.05$). However, there was no significant difference between the Zinc, Sepsis, and Sham groups. Additionally, no statistical difference was observed between the Hydroxychloroquine, Zinc, the group receiving hydroxychloroquine with zinc, and Sham groups.

Procalcitonin is a sepsis early biomarker which is expected to increase within 6–12 hours [24]. IL-6 is one another sepsis biomarker which is usually start to increase 1–2 hours after the insult [25]. In our study, blood have been collected 48 hours after cecum perforation. According our IL-6 and procalcitonin test results, there were no difference between sepsis and other groups. All histopathological and biochemical parameters' results were correlated with sepsis except IL-6 and Procalcitonin. This can be releated with the acute severity of the CLP model and limited sample size. The absence of differences in IL-6 and procalcitonin levels may be related to sampling time, acute mortality of the CLP model, or limited statistical power. One another possibility is IL-6 and Procalcitonin kits obtained from the same company and this makes us to think that there can be a non-functional kits possibility.

Despite the known zinc ionophore activity of hydroxychloroquine, the combined administration of zinc and hydroxychloroquine did not confer additional histopathological protection compared to monotherapy. This finding may be explained by several mechanisms. First, intracellular zinc homeostasis is tightly regulated, and excessive intracellular zinc accumulation may activate counter-regulatory pathways or induce cytotoxic effects, limiting its therapeutic benefit or re-regulate intracellular zinc level [26]. Second, hydroxychloroquine has been reported to impair lysosomal function and autophagic flux, processes that are critical for tubular epithelial cell repair following septic injury and this can compensate zinc positive effect (27). Therefore, while hydroxychloroquine may reduce inflammatory signaling, its inhibitory effects on cellular regeneration may offset potential benefits derived from increased intracellular zinc levels [10].

This study has several limitations. First, the high mortality rate inherent to the CLP sepsis model limited the sample size and restricted analyses to the acute phase of sepsis. Second, intracellular zinc concentrations were not directly measured, preventing confirmation of hydroxychloroquine-mediated zinc uptake at the cellular level. Third, the absence of oxidative stress markers and apoptotic pathway analyses limits mechanistic interpretation of renal protection. Finally, long-term renal outcomes and functional parameters such as creatinine clearance were not assessed. These limitations highlight the need for future studies incorporating functional renal outcomes and molecular assessments of zinc signaling pathways.

7. Conclusion

This experimental study suggests that despite the previously demonstrated positive effects of hydroxychloroquine and zinc in a sepsis model when given individually, when administered together in a sepsis model, hydroxychloroquine, even though it increases intracellular zinc concentration by creating pores in the cell membrane, does not result in any changes in sepsis-related organ damage. Further studies incorporating direct measurements of intracellular zinc, regenerative pathways, longer treatment duration and examining different organs are warranted to clarify the mechanistic basis.

Declarations

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Author Contribution

B.A.C and K.A.: conceived and designed the study, developed the methodology, supervised the experiments, performed data analysis and interpretation, drafted the manuscript, reviewed and edited the manuscript, oversaw the project, and approved the final version.E.G.D. and E.E. and U.U.: Conducted histological and immunohistochemical evaluations, conducted animal experiments, collected samples, performed histological and immunohistochemical analyses, curated the data, and contributed to the original manuscript draft.H.H.S and G.A.S.: conducted animal experiments, interpreted data, and contributed to manuscript writing.A.K. and M.H.E: Conducted all biochemical analysisU.D.: provided laboratory support, technical assistance, interpreted data

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Figures



b

Figure 1

a) Necrotic cecum b) Collection of blood samples c) Removal of organs.

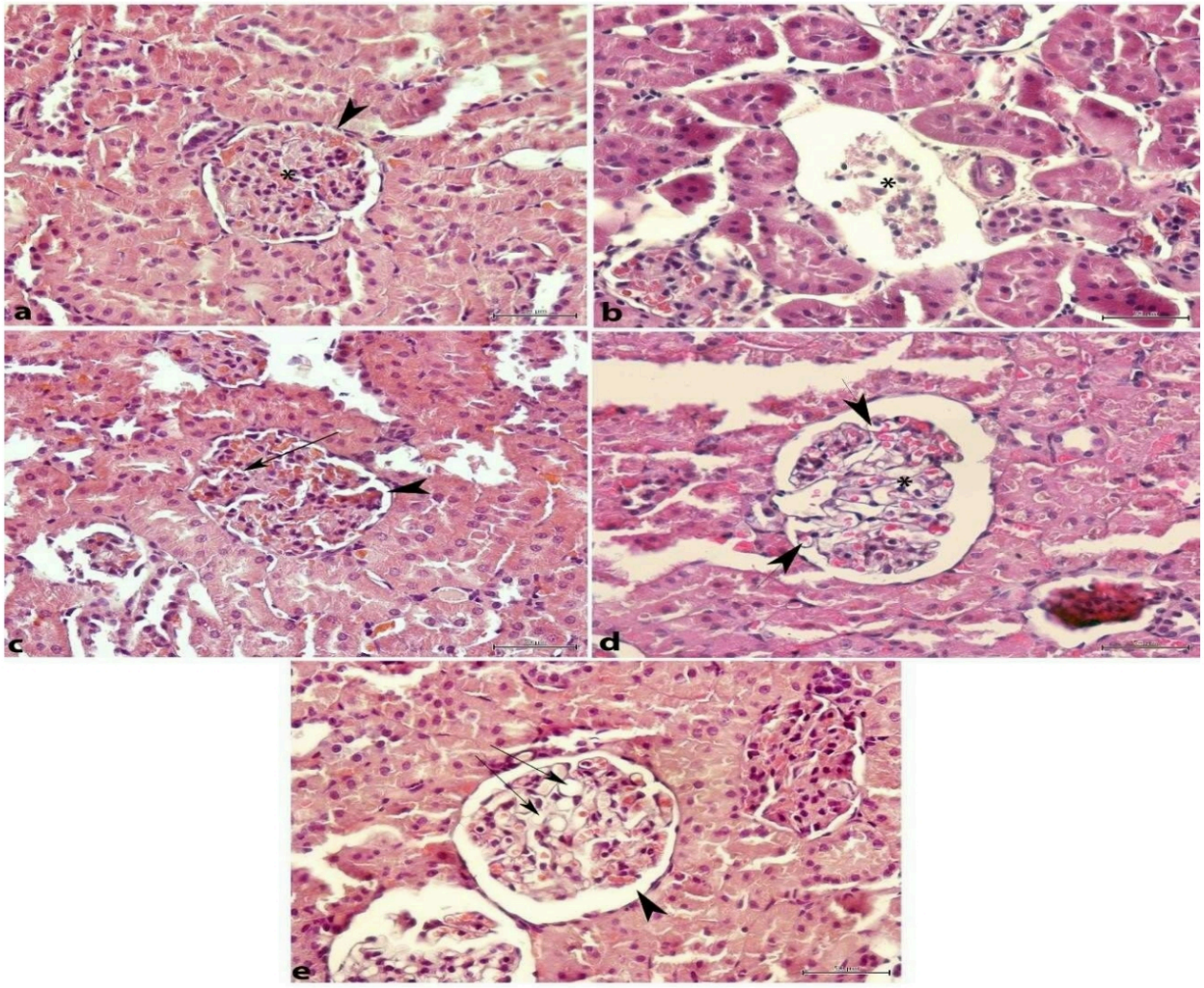


Figure 2

Histological micrographs of kidney glomeruli from sample groups in the experimental study.

a: This is a kidney glomerulus stained with H&E in the control group. The star symbol represents a healthy glomerulus, while the arrowhead indicates the Bowman's capsule. b: This is a kidney glomerulus stained with H&E in the sepsis group. The star symbol indicates glomerular atrophy, and the arrowhead shows Bowman's capsule enlargement c: This is a kidney glomerulus stained with H&E in the Zinc group. The arrowhead indicates Bowman's capsule enlargement, and the arrow symbol represents glomerular vacuolization. d: This is a kidney glomerulus stained with H&E in the Hydroxychloroquine group. The arrow symbol indicates glomerular vacuolization, while the star symbol represents glomerular atrophy. e: This is a kidney glomerulus stained with H&E in the group treated with Hydroxychloroquine along with zinc. The arrow symbols indicate vacuolization, while the arrowheads show Bowman's capsule enlargement.

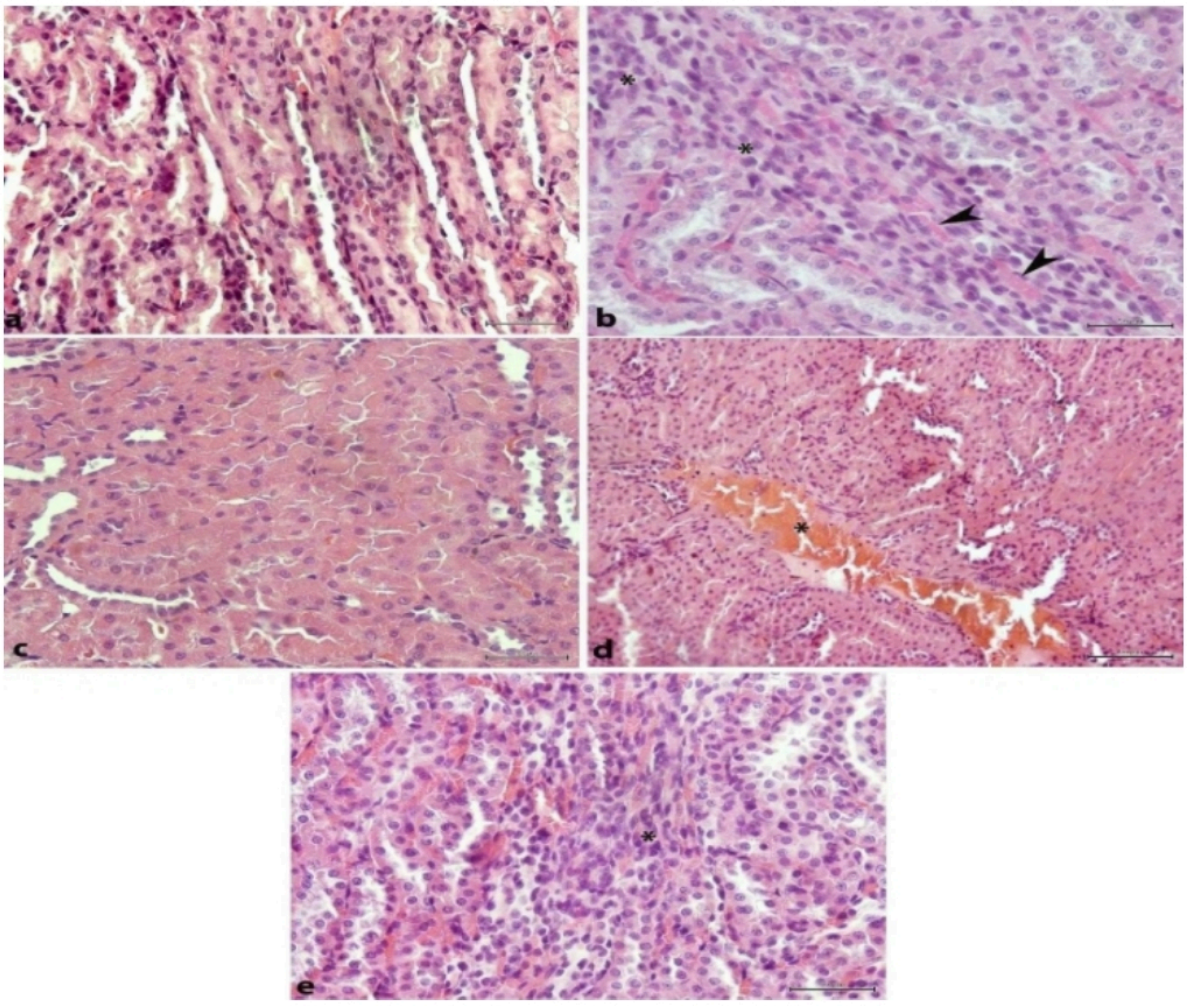


Figure 3

Histological micrographs of the renal interstitial tissue samples from the experimental groups.

a: Control group H&E-stained normal renal interstitial tissue b: The interstitium of the kidney tissue stained with H&E in the sepsis group shows arrowhead bleeding, indicating star sign mononuclear infiltration. c: Zinc group H&E-stained normal kidney interstitium. d: In the Hydroxychloroquine group, H&E stained interstitium reveals star sign bleeding. e: The interstitium stained with H&E in the group treated with Hydroxychloroquine and zinc together shows star sign bleeding and mononuclear infiltration.

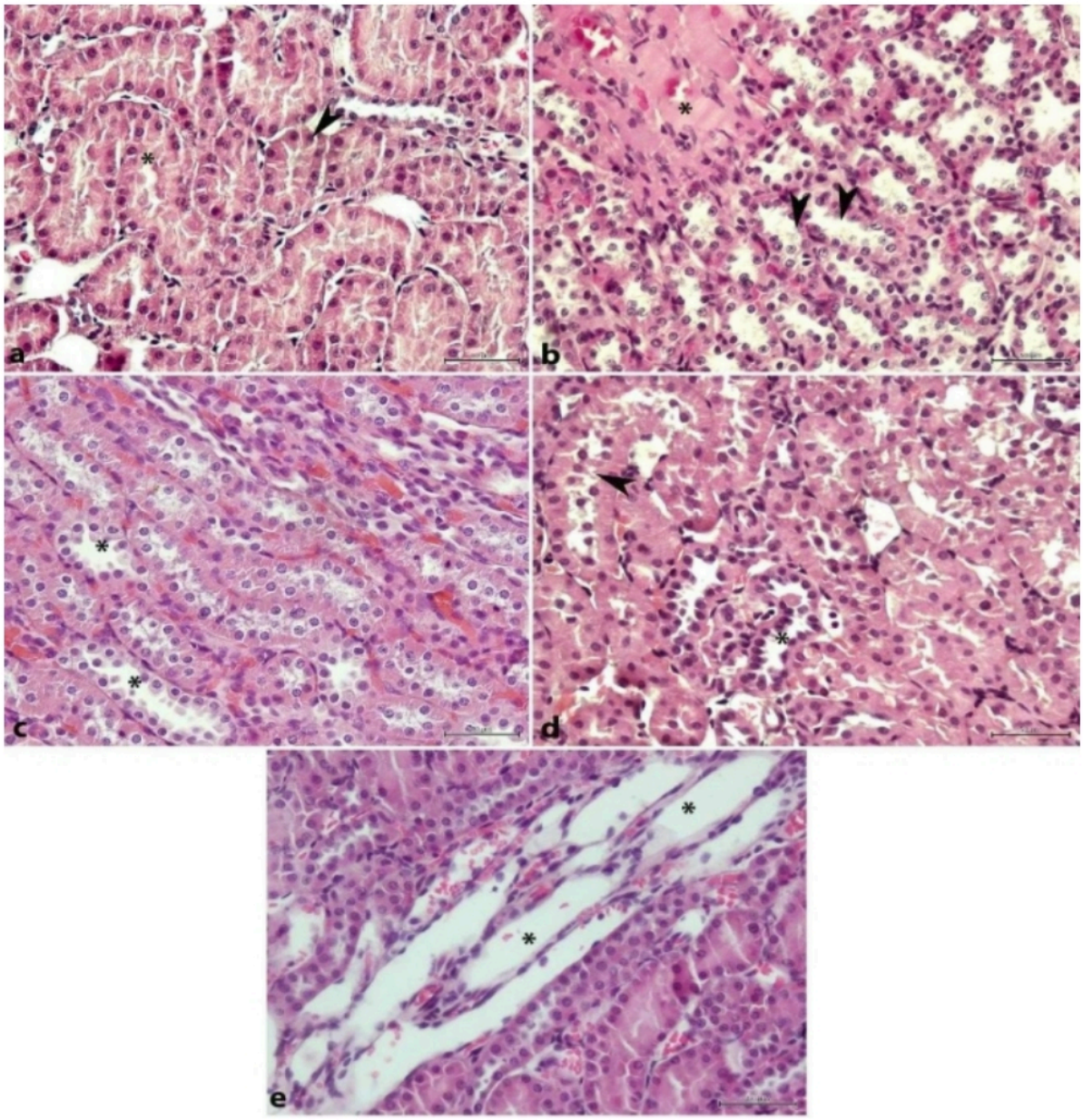


Figure 4

Histological micrographs of sample kidney tubules from the experimental groups.

a: Belonging to the control group, this is an H&E-stained kidney tissue, showing star sign healthy kidney tubules b: This is an H&E-stained kidney tubule belonging to the sepsis group, showing star sign cast formation, arrowhead tubular dilation, and necrosis. c: It is a kidney tubule belonging to the Zinc group, stained with H&E, showing star-shaped tubular dilation. d: Belonging to the tubule of hydroxychloroquine group which is stained H&E; star sign is dilated distal tubule arrowhead is dilated proxymal tubule e: This is a tubule of hydroxychloroquine+zinc group-stained H&E and star sign show tubuler dilatation