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The nephroprotective effect of amifostine in a cecal ligation-induced sepsis model in terms of oxidative stress and inflammation

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Abstract. – OBJECTIVE: Sepsis is responsible for more than 5 million deaths worldwide every year. The purpose of this study was to use amifostine to reduce acute kidney injury developing as a result of sepsis.

MATERIALS AND METHODS: Thirty Sprague Dawley rats were divided into three equal groups – a healthy control group (Group 1), cecal ligation and puncture group (CLP, Group 2), and a CLP + amifostine (AMF) group receiving a total of 200 mg/kg AMF intraperitoneally (i.p.) 15 min before sepsis induction (Group 3).

RESULTS: Total thiol levels decreased while malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), nuclear factor kappa B (NF-κB/p65), and interleukin (IL)-1β, and IL-6 levels increased in the CLP group. We also observed degeneration in renal corpuscles, necrotic tubules, polymorphonuclear leukocyte inflammation, and vascular congestion. In the amifostine group, total thiol levels in tissue increased, while MDA, TNF-α, NF-κB/p65, IL-1β, and IL-6 levels, necrotic renal tubules, and inflammation decreased.

CONCLUSIONS: Amifostine prevented sepsis-related acute kidney injury by reducing inflammation and oxidative stress.

Key Words:
Amifostine, Inflammation, Kidney, Oxidative stress, Sepsis.

Introduction

Sepsis represents the body's excessive and irregular reaction to infection, a life-threatening heterogeneous syndrome characterized by organ dysfunction and the main cause of infection-related deaths[1]. The incidence is approximately 20 million cases a year, its mortality rate is approximately 26%, and it is potentially responsible for more than 5 million deaths annually[2].

The importance of organ dysfunction is emphasized in the pathophysiology of sepsis, and such dysfunction plays a dominant role in sepsis-related mortality. The kidney is one of the organs damaged most frequently and earliest in sepsis, resulting in sepsis-associated acute kidney injury (S-AKI)[3]. AKI is present in approximately one in three critical septic patients[4]. At the same time, sepsis is the cause in 40-50% of patients developing AKI[5]. S-AKI is a life-threatening complication frequently seen in hospitalized and critical patients. S-AKI increases in-hospital mortality 6-8-fold and the risk of developing chronic kidney disease (CKD) 3-fold[6]. Additionally, one in four patients with S-AKI requires renal replacement therapy[7]. Renal injury is present at the time of the first presentation to the emergency department in approximately half of the patients developing S-AKI[8]. It therefore appears logical to regard sepsis as an early marker of S-AKI. The development of kidney injury in the early stages of sepsis limits the effectiveness of renal damage-preventing approaches. However, the fact that discharged sepsis patients with healed kidney damage exhibit similar one-year mortality to that of sepsis patients who have never developed kidney injury indicates that S-AKI is reversible to some degree[3,4]. Kidney functions improve in the majority of patients with the resolution of sepsis. Nonetheless, even a single attack of septic AKI is associated with an increased risk of future chronic kidney disease[9]. This represents a strong justification for the development of therapeutic strategies aimed at encouraging adaptive repair and reversing cellular damage for the prevention and early treatment of S-AKI.

A good understanding of the mechanisms of the underlying pathophysiology is essential for the prevention and early identification and treatment of S-AKI[10]. Although numerous animal and

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human studies[2,4-7,] have been performed to date, the mechanism by which sepsis leads to kidney injury is still not fully understood[11]. While it was formerly regarded as a hemodynamic disease caused by ischemia, recent research[12] suggests that S-AKI is a multifactorial disease whose pathogenesis also involves hemodynamic changes in the kidney, endothelial dysfunction, inflammatory cell infiltration into the renal parenchyma, intraglomerular thrombosis, decreased tissue oxygenation, and tubule obstruction by necrotic cells and wastes. Additionally, oxidative stress resulting from sepsis also contributes to the exacerbation of renal injury. Thiobarbituric acid reactive substances (TBARS) are an important marker of oxidative stress[13]. Previous studies[14] have also shown an increase in TBARS levels in S-AKI. Oxidative stress can be eliminated with endogenous glutathione (GSH), which exhibits antioxidant properties[15]. Studies[16] have also shown that decreased GSH levels deriving from S-AKI can be raised with the application of agents with antioxidant effects. Otherwise, increase oxidative stress precipitates inflammation in tissues[16].

Another important mechanism in sepsis-related renal injury is inflammation[17]. Studies[5] have shown an increase in proinflammatory cytokine levels in S-AKI. The production of proinflammatory cytokines, particularly interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), has a positive feedback effect on tissue damage[18]. In addition, transcription factors such as nuclear factor-kappa beta/mitogen-activated protein kinase (NF-κB/MAPK) constitute another important mechanism causing an inflammatory reaction in AKI[19]. Studies[18,20] have shown that NF-κB/p65 expression in the kidney triggers an oxidative and inflammatory effect, but that proinflammatory cytokine levels also decrease in case of inhibition. Inflammation also induces cellular apoptosis[21]. Reducing apoptosis is important to prevent the development of S-AKI.

Compared with non-septic AKI, S-AKI is thought to involve a different pathogenesis and to require a different approach[20]. It is therefore important to differentiate S-AKI from other acute kidney disease etiologies[3]. AKI can be diagnosed based on small changes in serum creatinine values and/or acute decrease in urine output. However, waiting for an increase in serum creatinine levels in the diagnosis of S-AKI may result in delays in diagnosis and treatment. Delayed diagnosis in S-AKI is associated with the worsening of clinical outcomes, such as the development of more advanced AKI, renal replacement therapy requirements, and greater chronic kidney disease in the long term[21].

There are powerful reasons for preventing S-AKI. That prevention relies on the treatment of sepsis and early resuscitation. However, the priority in this context is to identify high-risk patients. Such information is highly important to prevention and treatment planning.

Studies[4,7] have investigated and are still investigating the development of scoring systems and novel biomarkers to detect S-AKI at an earlier stage and improve clinical outcomes. Research[7] has focused on clinical risk assessment, novel kidney injury biomarkers, automatic electronic warnings embedded in electronic health records, and the concept of the Renal Angina Index. In addition, work has been performed on developing risk identification tools for adult patients in a critical condition, integrating such known risk factors as age, diabetes mellitus, heart failure, chronic kidney disease, liver disease, and malignancy together with potentially modifiable factors including fluid intake and urinary output[22]. However, our understanding of how these markers can be used in practice in the management of AKI is limited. Moreover, even if sepsis-related kidney injury is detected in the early stage, the measures that can be taken are currently limited. The importance of initiating anti-biotherapy against the source of infection in sepsis is beyond doubt[23]. Early and appropriate antimicrobial therapy in sepsis has been linked to a lower risk of AKI[24]. Every hour by which appropriate antimicrobial therapy is delayed increases the risk of AKI by approximately 40%. Additionally, early antimicrobial therapy is associated with a higher probability of kidney recovery within the first 24 h[24]. The results of studies[7] concerning the fluids and vasoactive agents used for resuscitation are inconsistent. In particular, there is significant evidence that starch-based and chloride-rich fluids may have an adverse impact on renal functions[25,26]. However, the effect of vasoactive drugs on kidney functions is variable[27]. Kidney functions may be irreversibly lost despite supportive therapies, and renal replacement therapy may be required[28]. However, data concerning when renal replacement therapy should be started and for how long it should be administered are insufficient[7]. A recent Phase II study[29-31] involving a small number of patients suggested that the administration of the endogenous phosphorylating enzyme bovine alkaline phosphatase (AP) may have beneficial

effects on renal functions in septic patients. However, there has been insufficient investigation of this. Unfortunately, no approved pharmacological therapy is currently available for S-AKI, probably due to the complex pathogenesis of the disease and our limited knowledge thereof[30]. Since only supportive care (such as fluid replacement therapy) can be provided for these patients, a search for a novel pharmacological therapy is required[29]. The discovery of agents with antiapoptotic effects capable of reducing oxidative stress and inflammation represents a source of hope for the development of new treatments[29-31]. In the context of that search[29-31] and based on the idea that the cytoprotective agent amifostine may also have a neuroprotective effect, we set out to investigate the effects on the kidney of amifostine in a cecal ligation-induced sepsis model in rats.

Amifostine (ethanethiol, 2-[(3-aminopropyl)]- dihydrogen phosphate(ester)) is an organic thiophosphate thought to act as a free radical scavenger[32]. Its use as a radioprotectıve and chemoprotective agent has been approved in several types of cancer[33]. In contrast to the agents used for cell protection in the clinical setting, amifostine is not specific to a single organ, but is an organic thiol compound capable of affecting all organs[34]. Amifostine has been reported[35] to exhibit a protective effect in preventing neutropenia, nephrotoxicity, ototoxicity, neurotoxicity, mucositis, and xerostomia associated with radiotherapy and chemotherapy. Studies[35] have determined that amifostine protects healthy tissue rather than ameliorating damaged tissue. This may be explained by the high capillary phosphatase activity in normal tissues and high pH value enhancing active thiol metabolite formation. Amifostine is not an active metabolite, but is converted into thiol, an active metabolite, in tissue by dephosphorylation with alkaline phosphatase[36]. The effective substance in tissue is a thiol. Amifostine exerts its cytoprotective effect in two ways; the first involves the scavenging of free radicals, while the second involves repairing DNA injury caused by free radicals by donating hydrogen ions[37]. Its polyamine-like structure and sulfhydryl group allow it to affect cellular processes and protect against the deleterious effects of chemotherapeutic agents and ionizing radiation[38]. Amifostine is also thought to exhibit antioxidant and cell-protective effects on the blood-gut barrier. However, only limited studies[39] have examined the effect of amifostine on bacterial translocation, one of which involved a radiation enteritis model.

The purpose of the present study was to investigate the nephroprotective effect of amifostine on AKI in rats using a sepsis model induced using cecal ligation.

Materials and Methods

Animal Study

The rats were divided into three groups of 10 animals each, with similar mean weights in each group. Group 1 (n=10), the healthy control group (CG), received only intraperitoneal (i.p.) 0.9% NaCl saline solution. Group 2 (n=10), the cecal ligation and puncture (CLP) group, underwent CLP-induced sepsis with no drug administration. Group 3 (n=10), the CLP + amifostine (AMF) group, received 200 mg/kg AMF *via* the i.p, route 15 min before sepsis induction with CLP[40].

Sepsis was induced using the sepsis model established in rats by means of CLP as previously described by Rittirsch et al[41]. All surgical procedures were carried out under sterile conditions. The rats were anesthetized with 90 mg/kg ketamine HCL injection and 10 mg/kg xylazine HCL. After confirming that the rats had been anesthetized, a mean 2.5-3 cm midline incision was made to the abdomen. The internal organs and cecum were identified through this small incision, and the cecum was sutured distally to the ileocecal valve using 3/0 silk sutures. Consistent with previous studies[40,42], two holes were made in the distal cecum, and contact was established between the cecal content and the peritoneum. Following washing with 1% lidocaine for wound analgesia, closure was performed with two layers of sterile silk 4/0 suture. The experiment was concluded 16 hours after the completion of the relevant procedures[43].

At the end of the experiment, the rats were euthanized with high-dose anesthesia. One of the kidneys was set aside for use in biochemical studies and stored at -80º C, while the other was placed into 10% neutral formalin.

Drugs and Chemicals

Amifostine (Ethyol 500 mg bottle) was obtained from Medimmun Pharma B.V. (Nijmegen, the Netherlands), and ketamine HCL (Ketalar) from Sanofi İlaç San. ve Tic. A.Ş. (Lüleburgaz/Kirklareli, Turkey), and xylazine (Rompun 2%) from Bayer Türk Kimya San. Ltd. Şti. (Istanbul, Turkey).

Biochemical Analysis

Tissue homogenization: A 1 L ph7.4 homogenization buffer was prepared with 20 mM sodium phosphate + 140 mM potassium chloride. Next, 1 mL homogenization buffer was added to 0.1 g tissue and homogenized in a Qiagen TissueLyser LT device (Qiagen, Venlo, Netherlands)[44]. Following homogenization, this was centrifuged at 800 g for 10 min. Total thiol (TT) group and TBARS assays were performed using the resultant supernatant.

Tissue cytokine levels (TNF-α) were measured using BT-LAB ELISA kits (Shanghai, China) in line with the manufacturer's instructions. The results were expressed as ng/g tissue.

TBARS assay

TBARS assay was performed as described by Ohkawa et al[45]. To 200 µL tissue supernatant was added 50 µL of 8.1% sodium dodecyl sulfate (SDS), 375 µL of 20 acetic acids (v/v) pH 3.5, and 375 µL 0.8% thiobarbituric acid (TBA). The mixture was then vortexed, and the reaction was left to incubate for 1 h in a boiling water bath. Following incubation, the mixture was cooled in ice water for 5 min and centrifuged for 10 min. The resulting pin color was read at 532 nm on a spectrophotometer.

Total Thiol (TT) content

Sedlak and Lindsay's spectrophotometric method was applied in the determination of total thiol groups[46].-SH groups were assayed using Ellman reagent. To 250 µL supernatant was added 1,000 µL 3M Na2HPO4 and 250 µL 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (4 mg DTNB was prepared in 1% 10 mL sodium citrate solution). After vortexing, absorbance at 412 nm was determined. The results were determined with a 1,000-62.5 µM reduced glutathione standard chart and were expressed as nmol/g.

Tissue cytokine (TNF-α) assay

Tissue cytokine levels were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits (BT LAB Cat. No. E0764Ra, Cat. Nos. E0119Ra and E0135Ra) using the sandwich ELISA immunoassay method.

Histopathological Analysis

Histopathological procedure

The extracted kidney tissues were buffered with phosphate buffer (pH: 7.4) in 10% neutral formalin solution (Sigma Aldrich, St. Louis, MO, USA) and kept for 36 h in a fixator. Following fixation, the tissue samples were subjected to routine tissue preparation procedures using a tissue processor (Citadel 2000, ThermoScientefic, Germany), including dehydration (with 50%, 70%, 80%, 90%, 96%, 100%, and 100% ascending ethanol series, Merck GmbH, Germany), clearing (xylol, Merck GmbH, Germany) and embedding in soft/hard paraffin (Merck GmbH, Germany). In the following stage, tissue samples embedded in hard paraffin (Merck GmbH, Germany) were placed in a rotary microtome (RM2525, Leica Biosystems, Germany) and sections 4-5 µm in thickness were taken. Following deparaffinization, the kidney tissue sections obtained were stained with Harris Hematoxylin (Merck GmbH, Germany) and Eosin G (Merck GmbH, Germany) using a staining device (Leica ST1050, Lecia Biosystems, Germany). The stained sections were then covered with Entellan (Merck GmbH, Germany) and photographed under a light microscope (BX51, Olympus Corp., Japan) with an attached digital camera (DP71, Olympus DP71, Olympus Corp., Japan).

Immunohistochemical analysis

NF-κB/p65 (ab32360, Abcam, UK) and IL-1β (ab283818, Abcam, UK) were used as inflammatory biomarkers, together with IL-6 primary antibodies (ab9324, Abcam, UK). Secondary kits compatible with the primary antibodies (Goat Anti-Rabbit IgG H&L, HRP, ab205718, Abcam, UK) were also employed. Primary and secondary antibody incubations were stained using the immunoperoxidase method with an IHC stainer (Bond Max III, Leica Systems, Germany) in line with the manufacturer's instructions. Finally, following incubation with primary and secondary antibodies, Harris hematoxylin (Merck, Germany) counterstaining was applied.

Semi-Quantitative Evaluation

Consistent with the histopathological findings from kidney tissue in previous studies[47-49] using experimental animal models, renal histopathological scores (RHS) for tissue sections stained with H&E were graded as shown in Table I based on degenerative renal corpuscles, tubular necrosis, polymorphonuclear (PMNL) inflammation, and vascular congestion findings. In addition, tissues exhibiting positive staining with primary antibodies using the immunoperoxidase method were graded according to the Immunohistochemical Staining Positivity Score described by Mercantepe et al[50] as shown in Table II. Scoring was performed under x20 and

Table I. Renal Histopathological Scores (RHS).

Score	Findings
Tubular Necrosis Score	
0	Less than ≤5%
1	Less than ≤25%
2	Less than ≤50%
3	Less than ≤75%
Degenerative Renal Corpuscles	
0	Less than ≤5%
1	Less than ≤25%
2	Less than ≤50%
3	Less than ≤75%
Interstitial Inflammation	
0	Less than ≤5%
1	Less than ≤25%
2	Less than ≤50%
3	Less than ≤75%
Vascular Congestion	
0	Less than ≤5%
1	Less than ≤25%
2	Less than ≤50%
3	Less than ≤75%

x40 magnification in 30 different randomly selected areas in each rat section by two histopathologists blinded to the experimental groups.

Statistical Analysis

Data elicited from biochemical and semi-quantitative analyses were analyzed on SPSS 18.0 software (IBM Corp., Chicago, IL, USA) using the Shapiro-Wilk's test, Q-Q plots, Skewness-Kurtosis values, and Levene's tests. Following normality tests, parametric data were calculated as mean±standard deviation. Differences between groups were evaluated using One-Way ANOVA and Tukey's HSD test. Non-parametric data were calculated as median and 25% - 75% interquartile range. Variations between groups were evaluated using the Kruskal-Wallis' test followed by Tamhane's T2 test. p-values ≤0.05 were regarded as statistically significant.

Results

Biochemical Analysis

TBARS levels measured to observe lipid peroxidation in kidney tissue increased significantly in the CLP group (Table III; $p<0.01$). TBARS levels were lower in the AMF group than in the CLP group (Table III; $p<0.01$).

Table II. Semi-quantitative Analysis.

Score	Findings
0	Less than ≤5%
1	Less than ≤25%
2	Less than ≤50%
3	Less than ≤75%

TT levels, measured to evaluate antioxidant activity in kidney tissue, were significantly lower in the CLP group compared to the CG (Table III; $p<0.01$). However, TT levels increased significantly in the amifostine group compared to the CLP group (Table III; $p<0.01$).

TNF-α levels in kidney tissues were higher in the CLP group than in the CG (Table III; $p<0.01$). However, TNF-α levels were lower in the AMF group compared to the CLP group (Table III; $p<0.01$).

Histopathological Analysis

Examination of the kidney tissue sections under light microscopy revealed normal renal corpuscles and proximal and distal tubule structures in the CG. One noteworthy finding was that the brush border in the proximal tubules also exhibited a typical structure [Figure 1 A-B, Table IV, RHS:1 (0-2)]. In contrast, widespread vacuoles in the cytoplasm of the degenerative renal corpuscle and renal tubular epithelial cells and accompanying necrotic tubules were observed in the CLP group. Vascular congestion and inflammation consisting of widespread polymorphonuclear leukocytes were present in the interstitial areas [Figure 1 C-D, Table IV, RHS: 9.50 (9-11)]. However, in the AMF application group, we observed a decrease in degenerative renal corpuscles, particularly necrotic tubules. In addition, we observed decreased inflammation and vascular congestion [Figure 1 E-F, Table IV, RHS: 3 (2-3)].

Table III. SBiochemical Analysis Results (mean ± standard deviation).

Group	MDA (nmol/gr tissue)	TT (nmol/gr tissue)	TNF-α (ng/g tissue)
Control	160 ± 26	7.26 ± 0.48	1.81 ± 1.7
CLP	210 ± 25[a,b]	6.8 ± 0.81	2.05 ± 2.18[d]
CLP+AMF	140 ± 8[b]	8.6 ± in1.16[c]	1.55 ± 0.15[b]

[a]$p\leq0.001$: *vs*. the control group. [b]$p\leq0.001$: *vs*. the CLP group. [c]$p\leq0.001$: *vs*. the CLP group. [d]$p\leq0.001$: *vs*. the control group. One-Way ANOVA/Tukey HSD.

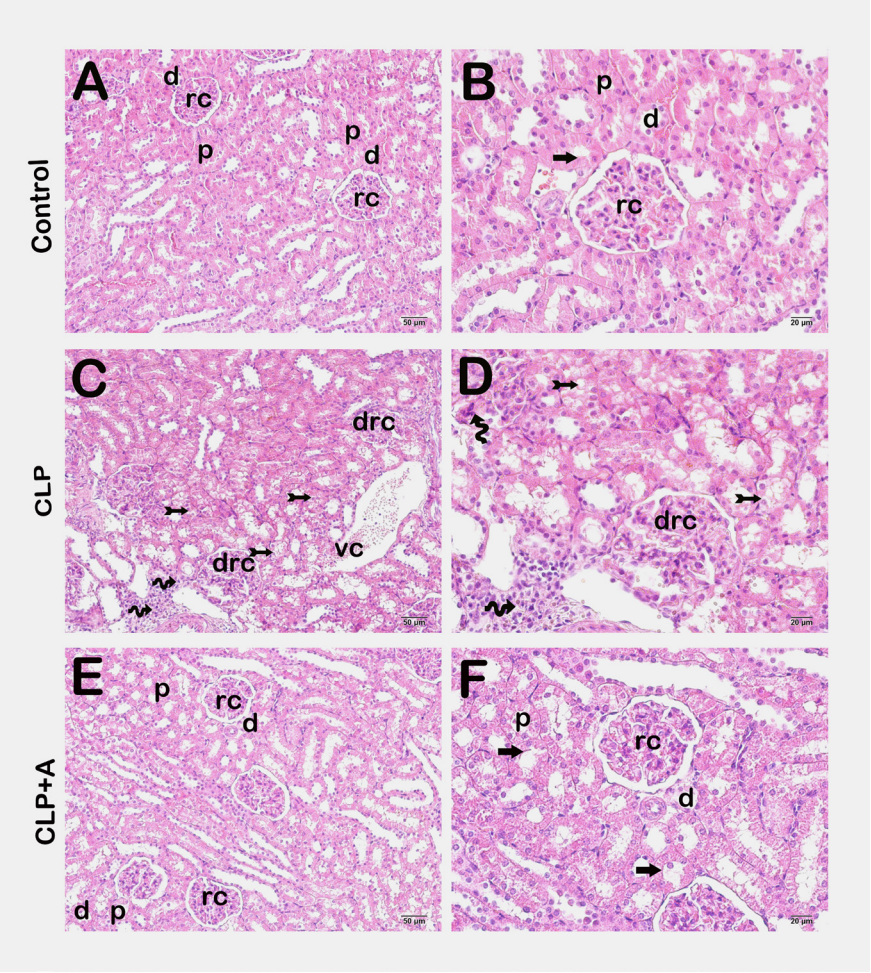

Figure 1. Representative light microscopy image of kidney tissue sections. Sections stained with Harris hematoxylin and Eosin G. Renal corpuscle (rc), Proximal tubules (p), Distal Tubules (d).
A-B, Sections from the control group exhibiting a normal renal corpuscle structure (rc) and renal tubules (p,d). The brush border (arrow) is particularly evident in the proximal tubules [RHS 1(0-2)]. A: x20, B: x40.
C-D, Widespread degenerative renal corpuscles (drc) and necrotic tubule structures (tailed arrow) characterized by epithelial cells with vacuoles in the cytoplasm can be seen in sections from the CLP group. Widespread inflammation (spiral arrow) consisting of polymorphonuclear leukocytes (PMNL) in the interstitial areas and vascular con gestions (vc) can also be seen [RHS 9.50(9-11)]. C: x20, D: x40.
E-F, Sections from the amifostine treatment group exhibit widespread typical renal corpuscles (rc) and renal tubules (p,d), with fewer degenerative corpuscles and necrotic tubules. Inflammation and vascular congestion can also be seen [RHS 3(2-3)]. E: x20, F: x40.

Semi-Quantitative Analysis

Examination of renal histopathological scores (RHS) calculated based on degenerative renal corpuscles, tubular necrosis, polymorphonuclear neutrophil inflammation, and vascular congestion revealed that the RHS value in the CG [1 (0-2)] increased significantly in the CLP application group [9.50 (9-11)] (Table IV; p=0.000). However, the RHS value in the CLP group [9.50 (9-11)] decreased significantly in the AMF group [3 (2-3)] (Table IV; p=0.000).

Table IV. Renal Histopathological Score (RHS) Results [median (25%-75% interquartile range)].

Group	Score				
	Tubular Necrosis Score	Degenerative Renal Corpuscle	Interstitial Inflammation Score	Vascular Congestion	RHS
Control	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-0)	1 (0-2)
CLP	3 (2-3)[a]	3 (3-3)[a]	2 (2-2)[a]	2 (2-2)[a]	9.50 (9-11)[a]
CLP+AMF	1 (1-1)[b]	1 (1-1)[c]	0 (0-1)[b]	0 (0-1)[b]	3 (2-3)[b]

[a]p=0.003, compared to the control group. [b]p=0.000, compared to the CLP Group. [c]p=0.000, compared to the CLP Group. Kruskal-Wallis'/Tamhane's T2 test.

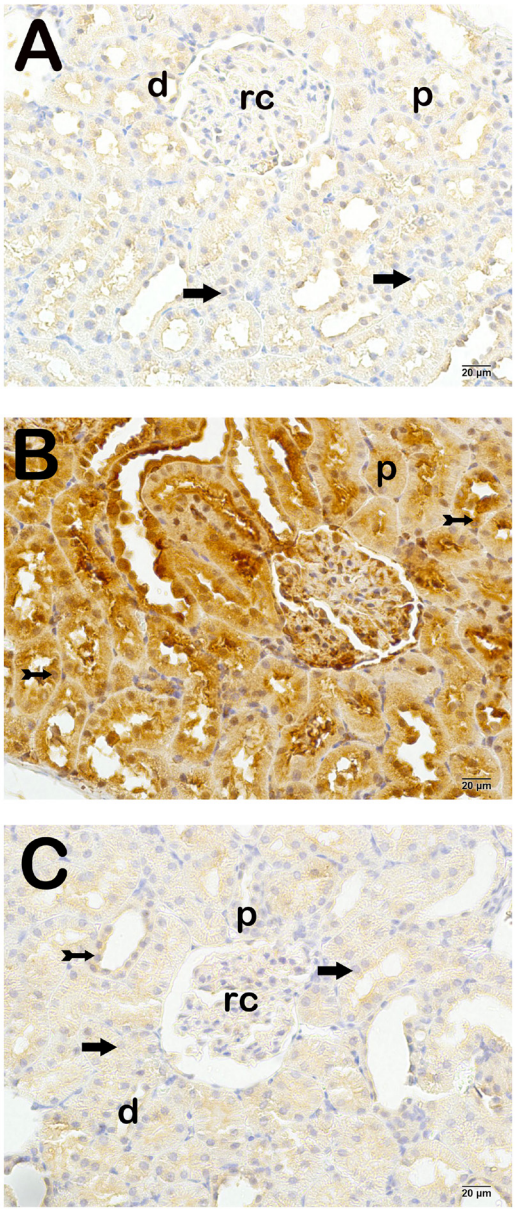

Figure 2. Representative light microscopy screen image of kidney tissue sections incubated with NF-κB/p65 primary antibody. Renal corpuscle (rc), Proximal tubules (p), Distal Tubules (d).
A, Kidney tissue sections from the control group exhibit a normal appearance [NF-κB/p65 positivity score 0 (0-1)] (x40).
B, Sections from the CLP group exhibit numerous renal tubule epithelial cells with NF-κB/p65 positivity [NF-κB/p65 positivity score 3 (3-3)] (x40).
C, A decrease can be seen in the numbers of renal tubule epithelial cells exhibiting NF-κB/p65 positivity in sections from the CLP+AMF group [NF-κB/p65 positivity score 1(1-1)] (x40).

Immunohistochemical (IHC) Analysis

Examination under light microscopy of kidney tissues incubated with NF-κB/p65 primary antibodies revealed that the NF-κB/p65 positivity score of 1 (0-1) in the CG increased to 3 (3-3) in the CLP group (Figure 2 A-B; Table V; p=0.000). In contrast, the NF-κB/p65 positivity score of 3 (3-3) in the CLP group decreased to 1 (1-1) in the AMF group (Figure 2 B-C; Table V; p=0.003).

Examination under light microscopy of kidney tissues incubated with IL-1β primary antibodies revealed that the IL-1 IL-1β positivity score of 0 (0-1) in the CG increased to 2 (2-2) in the CLP group (Figure 3 A-B; Table V; p=0.000). However, the, IL-1 IL-1β positivity score of 3 (3-3) in the CLP group fell to 1 (1-1) in the AMF group (Figure 3 B-C; Table V; p=0.000).

Examination under light microscopy of kidney tissue sections incubated with IL-6 primary antibodies revealed a higher IL-6 positivity score in the CLP group [2 (2-2)] than in the CG [0 (0-0] (Figure 4 A-B; Table V; p=0.000). However, the IL-6 positivity score of 2 (2-2) in the CLP group decreased to 1 (1-1) in the AMF group (Figure 4 B-C; Table V; p=0.000).

Table V. Semi-quantitative Analysis Results [median (25%-75% interquartile range)].

Group	NF-κB/p65 Positivity Score	IL-1β Positivity Score	IL-6 Positivity Score
Control	0 (0-1)	0 (0-1)	0 (0-0)
CLP	3 (3-3)[a]	2 (2-2)[a]	2 (2-2)[a]
CLP+AMF	1 (1-1)[b]	1 (1-1)[c]	1 (1-1)[c]

[a]p=0.000, compared to the control group. [b]p=0.003, compared to the CLP Group. [c]p=0.000, compared to the CLP Group. Kruskal-Wallis'/Tamhane's T2 test.

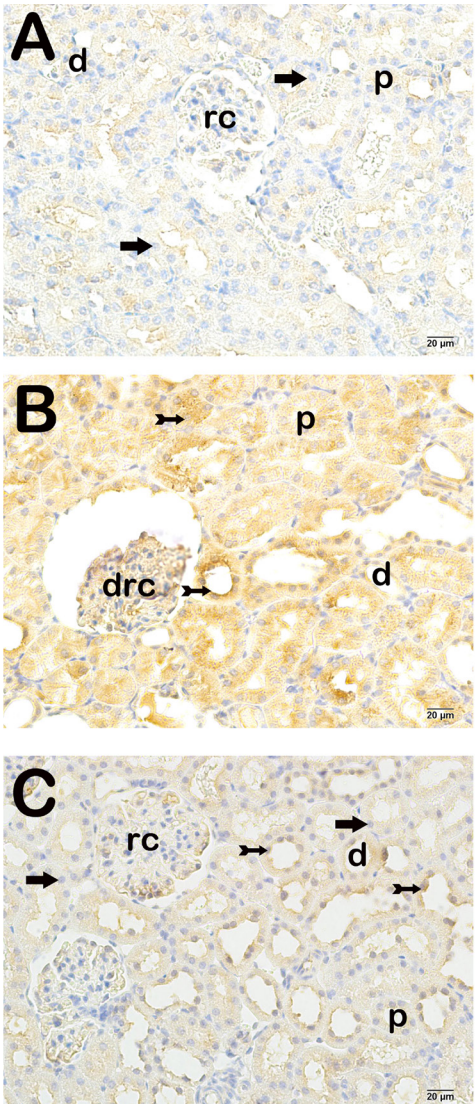

Figure 4. Representative light microscopy image of kidney tissue sections incubated with IL-6 primary antibody. **A**, Immune-negative renal tubule epithelial cells in kidney tissue sections from the control group [IL-6 positivity score 0 (0-0)] (x40). **B**, Numerous renal tubule epithelial cells exhibiting intense IL-6 positivity in sections from the CLP group [IL-6 positivity score 2 (2-2)] (x40). **C**, A decrease can be seen in the number of renal tubule epithelial cells exhibiting IL-16 positivity can be seen in kidney sections from the CLP+AMF group [IL-6 positivity score 1 (1-1)] (x40).

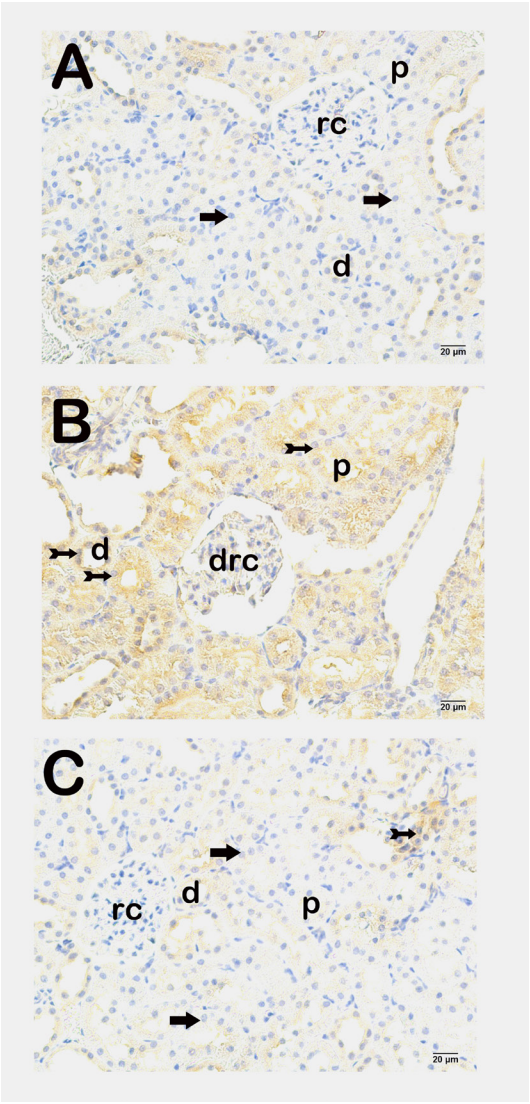

Figure 3. Representative light microscopy image of kidney tissue sections incubated with IL-1β primary antibody. **A**, IL-1β-negative renal tubule epithelial cells in kidney tissue sections from the control group [IL-1β positivity score 0 (0-1)] (x40). **B**, Numerous renal tubule epithelial cells exhibiting IL-1β positivity can be seen in sections from the CLP group [IL-1β positivity score 2 (2-2)] (x40). **C**, A decrease can be seen in the numbers of renal tubule epithelial cells exhibiting IL-1β positivity can be seen in kidney sections from the CLP+AMF group [IL-1β positivity score 1 (1-1)] (x40).

Discussion

This experimental study revealed the nephroprotective effect of amifostine in a sepsis model induced by means of cecal ligation. Early application of amifostine in situations involving a risk of sepsis appears to play an important role in reducing renal injury. S-AKI is an important problem with severe results contributing to morbidity and mortality. Although the mechanism underlying S-AKI is not yet fully understood, sepsis-related inflammation and oxidative stress play an important role in the pathophysiology. This study identified the relationship between renal damage and

inflammation by measuring inflammatory and oxidation markers. At the same time, the renal injury was determined using histopathological and immunohistochemical methods.

The presence of increased oxidative stress is regarded as playing a role at least as important as that of the increased inflammatory response in the pathogenesis of sepsis-related organ damage[51]. Levels of malondialdehyde (MDA), an important marker of oxidative damage, were measured in this study and decreased in the AMF group compared to the CLP group. MDA is a product of injury deriving from oxidative stress[52]. Previous studies[14,18,53] have shown that MDA levels rise in renal injury caused by sepsis and endotoxic shock. Similarly in the present study, MDA levels were significantly higher in the CLP than in the AMF and control groups. This is a finding showing that oxidative stress plays a significant role in the pathophysiology of S-AKI. MDA levels in the AMF group were significantly lower than in the CLP group. This was consistent with the results of studies[39,54,55] showing that amifostine exhibits an antioxidant effect against therapeutic agents. However, to the best of our knowledge, no previous studies have shown the inhibitory effects of amifostine against sepsis-associated inflammation and oxidation. However, the mechanisms underlying and antioxidant and anti-inflammatory effects of amifostine remain unclear.

GSH thiol (-SH) plays an important role in cellular defense against oxidative stress[56]. In the present study, we set out to determine antioxidant levels by measuring TT levels. TT levels in the AMF group were significantly higher than those in the CLP group. However, TT levels in the CLP group were significantly lower than those in the CG. The decreased TT levels in the CLP group in the present study were consistent with the findings of Xia et al[57] and Chen et al[58] studies. This finding suggests that amifostine may also exhibit antioxidant effects against S-AKI. However, no comparison was possible due to the absence of previous similar studies with amifostine.

NF-κB is the most important molecule triggering the synthesis of proinflammatory cytokines[17]. Increased free oxygen radicals (ROS) resulting from oxidative stress caused by cellular damage, trigger NF-κB-mediated inflammation and exhibit a positive feedback effect on one another[59]. Endothelial damage occurring in sepsis probably develops *via* the NF-κB pathway. The changes in TNF-α and NF-κB levels in the CLP and AMF groups in the present study were similar, and both were significantly higher in the CLP group than in the AMF and CG, thus supporting the idea of a relationship between the two. The decreased NF-κB /p65 positivity in the AMF group compared to the CLP group also suggests that amifostine may exhibit anti-inflammatory effects.

Previous studies[47,59,60] have shown that the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 increases in S-AKI. The precipitation of oxidative stress leads to an increase in proinflammatory cytokine production. Renal injury is inevitable if the inflammation is not brought under control. Similarly to the present study, many studies[14,18,53] showed an increase in the synthesis of these proinflammatory cytokines in S-AKI. TNF-α, IL-1β, and IL-6 levels in this research were significantly lower in the AMF group than in the CLP group. This in turn supports the hypothesis that these inflammatory processes and the TNF-α can be brought under control with amifostine.

The oxidative stress and inflammation deriving from sepsis also contribute to renal injury by inducing apoptosis[61]. Previous studies[61,62] have shown that apoptotic enzyme activity increases in S-AKI. Caspases are some of the most important biomarkers of apoptosis[63]. Increasing levels in tissue lead to the precipitation of apoptosis[64]. Caspase-3 levels also increased significantly in the CLP group compared to the AMF and CG in the present studies. Caspase-3 levels were lower in the AMF group than in the CLP group, and effectively reduced renal injury. This finding was consistent with the results of studies[36,39,54,55] investigating the radioprotective and chemoprotective effects of amifostine and showing that it protected healthy cells against apoptosis without affecting tumor cells. The anti-apoptotic effect of amifostine is in all likelihood mediated by hypoxia, by increasing the expression of various proteins involved in DNA repairs, such as Bcl-2 and hypoxia-inducible factor-1 (HIF-1)[65]. Amifostine certainly exhibits a selective antiapoptotic effect in healthy cells, without preventing apoptosis in tumor cells. This is due to tumor cells being relatively hypovascular and possessing low alkaline phosphatase activity, the enzyme needed for amifostine to be dephosphorylated to its active metabolite WR-1065. Endogenous alkaline phosphatase is ubiquitous, but principally present in epithelial cells. The function of the enzyme found in the brush border of the proximal tubule in the kidney is unclear, although it is partly responsible for phosphate reabsorption[29]. However, alkaline

phosphatase has been found to be upregulated in the kidneys during sepsis in experimental sepsis models. In their Phase 1 and Phase 2 studies, Heemskerk et al[30] showed that bovine alkaline phosphatase exhibits a nephroprotective effect in S-AKI. It may be speculated that both amifostine and endogenous alkaline phosphatase may exert a protective effect through similar mechanisms in S-AKI, either together, or by affecting one another. Future animal studies aimed at elucidating this mechanism might usefully be performed by comparing the two separately and in combination.

In their study of cecal ligation-induced kidney injury, Kostakoglu et al[47] observed that sepsis resulted in renal tubular necrosis and vascular congestion. In another study using a sepsis model induced by means of cecal ligation, Melo Ferreira et al[49] reported that this caused neutrophil infiltration in the renal cortex and medulla. Malkoç et al[48] reported that sepsis induced by means of cecal ligation resulted in diffuse edematous areas, inflammation, and vascular congestions. Cai et al[66] demonstrated that cecal ligation-induced renal tubular necrosis. Jiang et al[67] reported damage in renal tubules as a result of AKI induced with cecal ligation. Similarly in the present study, cecal ligation-induced sepsis was found to result in polymorphonuclear leukocyte infiltration and vascular congestion. We also observed necrotic tubules and degenerative renal corpuscles.

Limitations

There are a number of limitations to this study, including the fact that serum blood urea nitrogen (BUN), creatinine, and urine levels could not be measured. However, this research is an important pilot study showing the antioxidant and anti-inflammatory effects of amifostine in sepsis-related kidney injury.

Conclusions

Amifostine reduced renal damage resulting from sepsis through its antioxidant, anti-inflammatory, and antiapoptotic effects. Amifostine may thus open a door to promising studies regarding the prevention of S-AKI. However, serum BUN, creatinine, and urine levels need to be measured and their clinical effects examined in future studies. It will also be logical to investigate the effects on the nephroprotection of amifostine and alkaline phosphatase together.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Authors' Contributions

All authors take public responsibility for the content of the work submitted for review. The authors confirm their contribution to the paper as follows: Sule Batcik and Levent Tumkaya designed this study, conceptualization, investigation, formal analysis, writing - of the original draft, and supervision. Sule Batcik, Levent Tumkaya, Tolga Mercantepe, Atlla Topcu: investigation. Tolga Mercantepe: investigation, formal analysis, writing - original draft. Mehtap Atak, Huseyin Avni Uydu: formal analysis, investigation. Filiz Mercantepe: writing. The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

Ethics Approval

This study was approved by The Research Ethics Committee of the Medical Faculty, Rize Recep Tayyip Erdogan University (Approval Number: 2020/12, Approval Date: 02/06/2020). The study was carried out according to the principles of the Declaration of Helsinki.

Availability of Data and Materials

The datasets in the current study are available from the corresponding author upon reasonable request.

Informed Consent

Not applicable.

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