ORIGINAL ARTICLE OPEN ACCESS

Renal Protective Effect of Naringin in an LPS-Induced Acute Kidney Injury Model in Mice

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ABSTRACT

Background: Acute kidney injury (AKI) is a severe consequence of sepsis, characterized by rapid onset and high mortality. Naringin has broad pharmacological effects; however, its role in AKI remains unclear. Here, we evaluated the protective effects of naringin against lipopolysaccharide (LPS)-induced AKI in mice.

Methods: This in-vivo pre-clinical experimental study was conducted at Ziauddin University, from March-October 2023. Using Simple Random sampling, 24 male BALB/c mice weighing between 25-30 grams were divided into 4 groups (n=6). Normal control group (A), received intraperitoneal (I.P.) normal saline; Disease group (B) received LPS (2mg/kg; I.P.); The naringin-treated groups (C&D) were subjected to I.P. naringin treatment at 50mg/kg and 100 mg/kg doses for 4 days followed by 2mg/kg LPS I.P. Serum creatinine and urea levels were measured using calorimetric assay and kidney histopathological changes by H&E & PAS. Using SPSS v.25, Shapiro-Wilk test was used for normality and ANOVA, and Tukey's Post Hoc analysis was done for inter-group comparison.

Results: LPS induced elevation of serum creatinine and urea, but naringin treatment significantly reduced these (p=0.000 and p=0.026). LPS also increased tubular injury scores. Naringin significantly reduced LPS-induced Tubular injury (p=0.026). Group-C showed a significant difference from normal, indicating disease development. Conversely, the 100mg/kg group not only distanced itself from the disease but also approached normal levels showing that improvement is more pronounced in the 100mg/kg.

Conclusion: Naringin protects renal tubular cell morphology from LPS-induced damage. Due to this effect, it preserves the urea and creatinine clearance functions of these cells, ultimately preventing acute renal failure.

Keywords: Lipopolysaccharide (LPS), Inflammation, Acute Kidney Injury, Sepsis.

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Doi: 10.36283/PJMD13-3/014

How to cite: Shaikh S, Hameed A, Bhagwani A R, Borges K J J Renal Protective Effect of Naringin in an LPS-Induced Acute Kidney Injury Model in Mice. Pak J Med Dent. 2024;13(3): 98-105. Doi: 10.36283/PJMD13-3/014

Received: Sat, Apr 27, 2024 **Accepted:** Thu, June 06, 2024 **Published:** Wed, 24 July 2024

INTRODUCTION

Severe sepsis is a critical condition that impacts a large number of critically ill patients, especially those who have undergone surgery. sepsis-associated AKI leads to a worse prognosis than sepsis or acute kidney injury alone. It is linked to longer stays in the intensive care unit (ICU) and hospital, higher mortality rates, increased long-term disability, and reduced quality of life in both adult and pediatric patients[1] . Acute kidney injury occurs in 10%-15% of hospitalized patients and 50% in the intensive care unit setting[2] . The mortality rate for critically ill patients with sepsis-associated acute kidney injury ranges from 38.2 to 70.2 % [3]. Sepsis-induced acute kidney injury causes a rapid decline in renal function particularly in the glomerular filtration rate, within a short period. This rapid deterioration makes it difficult to maintain fluid and electrolyte balance[4]. Sepsis often presents with signs and symptoms involving multi-organ systems. the extensive release of inflammatory mediators during sepsis can result in multi-organ failure, necessitating its management as a systemic disorder[5]. For a long time, ATN caused by hypoxia combined with a severe hyperinflammatory response was considered the primary cause of renal failure in patients with sepsis-associated acute kidney injury[6].

Acute kidney injury (AKI) can result from various events that are harmful to the kidneys, including reduced blood flow to the kidneys (renal hypoperfusion), blockage of the urinary system, rapidly worsening glomerulopathy, sudden inflammation of blood vessels (acute vasculitis), and sudden inflammation of the kidney tissue (acute interstitial nephritis). Of all the conditions, sepsis is the most prevalent[7]. The development of AKI due to sepsis is also affected by acute tubular necrosis and malfunction of tubular epithelial cells AKI development is characterized by elevated blood urea nitrogen and serum creatinine levels, along with a decline in glomerular filtration rate[8].

Sepsis is highly consequential in the intensive care unit (ICU), impacting over 30% of patients, with substantial differences across different geographical regions[9]. In high-income nations, the occurrence of sepsis is estimated to be around 30 million cases per year, and it is linked to an annual death rate of 5.3 million[10]. In low-middle-income countries, hemodialysis is often scarce and expensive presenting challenges in providing this treatment. As an alternative, peritoneal dialysis is frequently seen as a more effective and affordable option though it is usually limited to short-term use during the acute phase. The quality of care for patients with acute kidney injury has been a concern. while preventive treatments have shown benefits for specific causes of AKI such as radiocontrast–induced and post-cardiac surgery AKI, the most effective strategies for preventing AKI remain a topic of debate[11]. Given these findings, it becomes imperative to concentrate on targeted therapy for sepsis-associated acute kidney injury to ameliorate adverse outcomes. Patients with SA-AKI seem to face a disadvantage, in part due to their elevated level of illness severity[12].

Lipopolysaccharide, an endotoxin obtained from the outer membrane of Gram-negative bacteria, is commonly employed to induce sepsis and create an animal model of Sepsis-AKI[13]. Sepsis leads to the release of LPS into the bloodstream, where it attaches to Toll-like receptors (TLRs). This triggers many signaling pathways that contribute to the failure of multiple organs[14] . LPS, a major contributor to sepsis, plays a critical role in the development of acute kidney injury. It induces excessive inflammatory responses, which in turn escalate oxidative stress, renal hypoperfusion, and serve kidney damage [15]. Thus, animal models of LPS-induced AKI are widely used for the study of new compounds.

Naringin, a widely recognized flavanone glycoside is found in grapes and citrus fruits. It exhibits diverse biological properties, including antioxidant, antibacterial, anti-atherosclerosis, anti-inflammatory, and hypoglycemic effects[16]. Nevertheless, the influence of naringin on LPS-associated acute kidney injury has not been examined. Thus, in the current study endeavor, we explored the preventive effects of naringin on LPS-induced AKI.

METHODS

This in-vivo pre-clinical experimental study was conducted at Ziauddin University, from March-October 2023. Using simple random sampling, 24 male BALB/c mice weighing between 25-30 grams were divided into 4 groups of 6 each[17]. Underweight, overweight, or unhealthy mice and female mice were not included. Mice were maintained and kept in the animal house facility of Ziauddin University where they were kept in conventional cages at 22-23°C with a 12-h light-dark cycle. The animals were allowed free access to water and rodent chow. The animals underwent a period of acclimatization to the habitat before commencing the experiments. All experiments were conducted with prior approval from the Ziauddin University Animal Ethics Committee (AEC) Protocol No: (2023-03/SS/FHS).

Lipopolysaccharides (LPS), Dimethyl sulfoxide (DMSO), and naringin were purchased from Sigma. Isopropanol, acetone, and xylene were purchased from Merck. Hematoxylin and eosin solutions were purchased from Sigma. Urea and creatinine kits were purchased from Merck. Male BALB/c mice (n=24) were purchased from the animal house of Panjwani Center for Molecular Medicine (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

The 24 male BALB/c mice were randomly allocated

 DOI: https://doi.org/10.36283/PJMD13-3/014

into the following groups of 6 mice each; the vehicle used was saline. In Group 1: Animals were given a standard volume of vehicle (saline) intraperitoneal (I.P.), Group 2: (Disease model) Animals were given vehicle I.P. for 4 days and a single LPS injection of 2 mg/Kg I.P. on day 5. Group 3: (Pre-treatment low dose + disease induced) Animals were given Naringin 50 mg/Kg I.P. for 4 days and a single injection of LPS 2 mg/Kg I.P. on day 5. Group 4: (Pre-treatment high dose + disease induced) Animals were given Naringin 100 mg/Kg I.P. for 4 days and LPS 2mg/Kg I.P. on day 5 following the final dose of naringin. Following 24 hours, the mice were euthanized, blood was obtained through heart cardiac puncture and kidney tissues were removed for histological study.

The collected blood was allowed to stand for 20 minutes, followed by centrifugation at 2000 rpm for 10 minutes. Subsequently, blood creatinine and urea nitrogen levels were measured using an automated biochemical analyzer, Micro Lab 300 (ELITech). The harvested kidney tissues were placed in 10% formaldehyde overnight. On the next day, the tissues underwent dehydration using progressively higher concentrations of alcohol, cleared with xylene, embedded in paraffin, and molded into tissue blocks, 4µm sections were taken onto glass slides. For staining with H&E and PAS, the initial steps were deparaffinizing and rehydrating the tissues. The sections were then stained with relevant dyes after which they were dehydrated again, cleared with xylene and finally mounted with a coverslip using DPX media. The slides were examined under a microscope and histological changes including Tubular Injury Score {(number of damaged tubules / total number of tubules per high power field (400x) x 100)} in percentage calculated.

The data was entered into the SPSS version 25 to be analyzed. The quantitative variables were expressed as mean and standard deviation. The Shapiro-Wilk test was utilized to evaluate Normality. ANOVA and Tukey's Post Hoc analysis were employed for group comparisons. A significance level of $p<0.05$ was deemed statistically significant.

RESULTS

The naringin effect on the serum urea and creatinine in the LPS-induced AKI model in mice was evaluated to explore the potential protective impact of naringin on lipopolysaccharide-induced acute kidney injury. Shapiro-Wilk was applied to check the normality of urea, which was found to be insignificant in control, LPS, Naringin 50mg/kg as well as in Naringin 100mg/kg ($p=0.210$, 0.245, 0.713, 0.110 respectively). Similarly, Shapiro-Wilk for creatinine was found to be insignificant for control, LPS, Naringin 50mg/kg as well as Naringin 100mg/kg ($p= 0.460$, 0.420. 0.944 & 0.713 respectively). For the Tubular injury score, Shapiro-Wilk was again not significant in all groups ($p=0.5400$, 0.5805, 0.652, 0.110 respectively).

To compare urea levels between the groups, one-way ANOVA was applied which was statistically significant ($p=0.026$). Intergroup comparison showed that the serum urea of group B (65.98±20.13) was significantly higher than that of group A (38.47±10.74) indicating proper development of the model. Group D (39.2±6.28) showed significantly reduced urea levels as compared to Group B (65.98±20.13). Comparison between other groups was not significant. Similarly, one-way ANOVA was applied which showed a significant difference between the groups for creatinine levels. Intergroup comparison showed that the creatinine group B) (2.4±0.73) was significantly higher than that of group A (0.47±0.33) and both the treatment groups (C&D) showed significantly improved creatinine as compared to the disease group. (C=0.571±0.25 and D=0.48±0.28). The differences between other groups were not significant (Table 1).

Table 1: Effects of naringin on serum urea levels in LPS-induced AKI mice

Groups		Urea mg/dl (Mean±S.D)	Creatinine mg/dl (Mean±S.D)
A	Control	38.47±10.74	0.47±0.33
B	LPS	65.98±20.13	2.4±0.73
C	Naringin 50mg/kg	51.5±22.12	0.571±0.25
D	Naringin 100mg/kg	39.2±6.28	0.48±0.28
ANOVA (p-value)		0.026	0.000

One way ANOVA test was applied, p <0.05 was considered significant

Table 2A: **Comparison of Urea levels among different groups.**

Groups	Significant intergroup Comparison (p <0.05)
A (Control) - B(LPS)	(p<.037)
B (LPS)-D (Naringin 100mg/kg)	(p<0.44)

Table 2B: **Comparison of Creatinine levels among different groups.**

Groups	Significant intergroup Comparison (p <0.05)
A (Control) - B(LPS)	(p<0.000)
B (LPS) -C (Naringin 50mg/kg)	(p<0.000)
B (LPS) - D (Naringin 100mg/kg)	(p<0.000)

One Way ANOVA test was applied, and p <0.05 was considered significant

The histological alterations in kidney tissue were analyzed to assess the protective effects of naringin against LPS-induced acute kidney injury (AKI). Vehicle control group (A) showed normal tubular and glomerular renal structures after H&E staining. The LPS-treated group showed histological anomalies, including renal tubular vacuolation and tubular cell necrosis; however, both low and high doses of naringin pretreatment were found to attenuate LPS-induced renal damage. Specifically, the higher-dosage group showed a more pronounced improvement in kidney damage as compared to the lower-dosage group (Figure 1-A-D). H&E images of kidney sections reveal significant detachment of the epithelium from the basement membrane in the LPS group as compared to normal controls. (Figure-1A-B). Visible protection of nephron architecture can be observed in the treatment groups which is more prominent in the Nar-100mg group

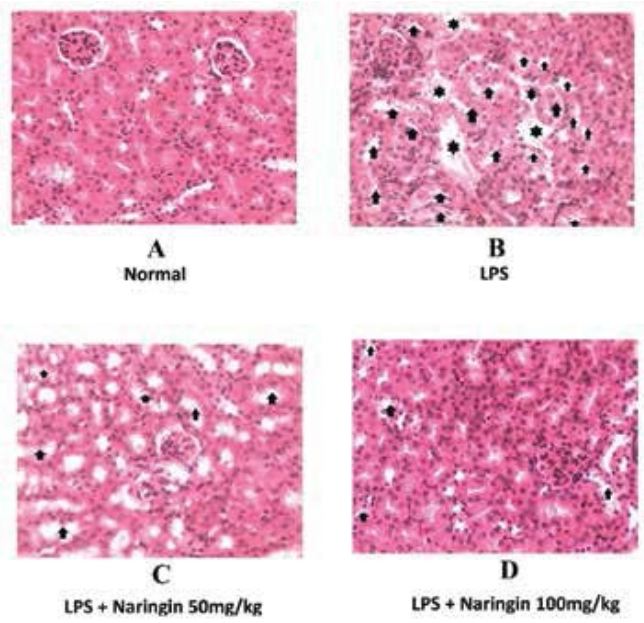


Figure 1: **Effects of naringin on histopathological deformities in kidney tissue of LPS-induced AKI mice H&E staining of the kidney tissues (A-D) (H&E staining, original magnification: 400X): A→Normal renal tubules with intact architecture and prominent brush border indicated by arrows. B→ shows LPS-induced damage to the renal tubules. Stars indicate necrotic areas whereas arrows indicate damage to the tubular epithelium. (C&D) demonstrates the effect of Nar-50mg/kg showing considerably less damage to the tubules as indicated by arrows.**

 DOI: https://doi.org/10.36283/PJMD13-3/014

Using PAS staining, the histological changes in the kidneys caused by LPS were assessed. One day after the injection of LPS, it was evident that there was renal pathologic damage, as evidenced by destruction and distention of tubular structures, elongation of Bowman's space, and, loss of tubular brush boundaries by staining the glycocalyx of the proximal tubule brush border. In LPS-injected mice, the PAS-stained cells were noticeably decreased but increased by naringin treatment both at low and high doses.

To quantify the histological anomalies, a tubular injury score was carried out on PAS-stained slides. Tubules tubular cast formation, tubular atrophy, tubular dilation, degeneration, vacuolization, loss of the brush border or thickening of the tubular basement membrane, sloughing off the tubular epithelial cells, were considered to be damaged and their percentage by the total number of tubules in the field at 400x magnification was calculated. A significant difference was observed between the normal groups (7.7% ± 4.5) and the AKI group (86.6 ± 2.3). Significant reduction in the score of treatment groups was observed in Nar-50mg scoring at (40 ± 5.2) and Nar-100mg at (13.7 ± 4.55) compared to the LPS group. (Table 3A)

Table 3A: Renal Tubular Injury Score Effects of Naringin on Tubular Injury Score in LPS-induced AKI Mice.

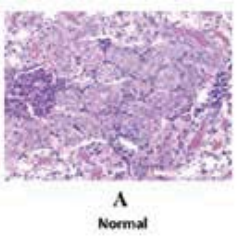
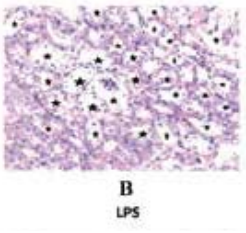
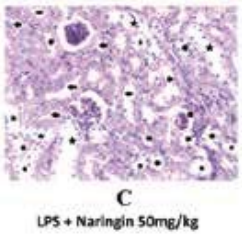
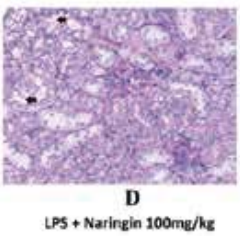
Groups		Tubular Injury Score (%) (Mean±S.D)	PAS Staining
A	Control	7.711±4.51	 A Normal
B	LPS	86.58±2.31	 B LPS
C	Naringin 50mg/kg	39.97±1.65	 C LPS + Naringin 50mg/kg
D	Naringin 100mg/kg	13.65±4.64	 D LPS + Naringin 100mg/kg
ANOVA (p-value)		0.026	

Table 3B: Comparison of Tubular Injury Score among different groups.

Groups	Significant intergroup Comparison (p <0.05)
A (Normal) – B (LPS),	(p<0.0001)
A (Normal) - C (Naringin 50mg/kg),	(p<0.0001)
B (LPS) - C (Naringin 50mg/kg),	(p<0.0001)
B (LPS) – D (Naringin100mg/kg),	(p<0.0001)
C (Naringin50mg/kg) - D (Naringin 100mg/kg)	(0.0003)

One Way ANOVA test was applied, and p<0.05 was considered significant.

DISCUSSION

Sepsis is a condition characterized by the body's extreme response to an infection by bacteria, viruses, parasites, or fungi, leading to damage to healthy tissues[18]. Patients who have acute kidney injury (AKI) associated with sepsis are at a significantly higher risk of mortality, with a six to eight times greater likelihood of dying compared to patients who have sepsis alone[19]. Sepsis is the primary etiology of acute kidney damage (AKI) in severely unwell individuals. This syndrome is distinguished by intricate pathophysiological pathways, encompassing inflammation, oxidative stress, and apoptosis[20]. The initial management of acute kidney injury focuses on correcting fluid balance and biochemical abnormalities, such as hyponatremia, hyperkalemia, and acidosis, which can be life-threatening. Preventing further kidney damage involves maintaining adequate blood pressure and avoiding nephrotoxic substances[21].

Prior research has demonstrated changes in serum creatinine and urea concentrations in the cisplatin-induced acute kidney injury model, alongside alterations in the expression of inflammatory factors in kidney tissue and serum. Additionally, renal pathology was assessed through H&E and periodic acid Schiff staining. The findings revealed a notable decrease in serum creatinine and urea nitrogen levels in the AKI model three consecutive days of intraperitoneal quercetin injection, indicating a significant improvement in renal function. Quercetin also mitigated tubular necrosis in the kidneys affected by AK[22].

Another study has shown that LPS increases the levels of creatinine and blood urea nitrogen. To investigate the therapeutic effects of oleuropein on acute kidney injury mice were treated with LPS via intraperitoneal injection. The result showed that LPS increased the kidney index causing damage and enlargement of the kidneys which was significantly reversed by OP treatment. OP treatment significantly alleviated the excessive increase in urea and creatinine levels induced by LPS. Additionally, histological analysis revealed that LPS triggered swelling and deformation of renal tubular epithelial cells and disrupted the normal kidney tissue structure, while OP treatment prevented LPS-induced acute kidney injury [23].

Naringin, a flavonoid glycoside primarily obtained from fruits and vegetables, is renowned for its potent antioxidant and anti-inflammatory properties[24]. Naringin can reduce COVID-19-induced lung injury by inhibiting interleukin-6 (IL-6) and also block LPS-induced pulmonary edema by suppressing the secretion of myeloperoxidase, TNF-alpha, and neutrophil infiltration[25]. Naringin has been recognized for its various biological functions, particularly its anticancer properties. It inhibits cell proliferation in several human cancer cell lines, including those of the stomach, colon, pancreas, breast, liver, and lung. Additionally, naringin can prevent angiogenesis, induce apoptosis in cancer cells suppress the release of tumor necrosis factor, and prevent hepatocellular damage caused by toxins like LPS by inhibiting protein kinase. known as a suppressing and blocking agent in cancer control, naringin halts the growth of new cancer cells and pathways initiated by carcinogenic compounds[26].

In our study, we have attempted to evaluate the protective effects of naringin on LPS-induced kidney injury. According to the reported data, and our experimental evidence in this research reveals that the blood urea and creatinine are markedly increased in the LPS-injected mice, evident as a key player in acute kidney injury[27]. However, after employing the naringin, both blood urea and creatinine levels were significantly reduced suggesting the promising role of naringin in the LPS-induced AKI protection. The kidney tissues' histological examination revealed, distinct deformities after the LPS induction. In the naringin treatment group, these cells are preserved. This shows that Naringin has the property of preserving renal tubular architecture against LPS-induced injury.

These results highlight the potential of naringin as a therapeutic candidate for the protection of renal damage caused by sepsis-induced AKI and could be a candidate drug for hospitalized patients. These

 DOI: https://doi.org/10.36283/PJMD13-3/014

findings offer promising implications for naringin's potential in sepsis treatment and lay the groundwork for future research on acute kidney injury mechanism(s). However, further investigation is needed to elucidate naringin's specific target in the TLR4-TNFα signaling pathway or the evaluation of any other target necessary. The limitation of this study is molecular pathways should be studied.

CONCLUSION

Naringin protects renal tubular cell morphological changes such as renal tubular vacuolation, tubular cell necrosis, destruction and distention of tubular structures, and loss of brush border from LPS-induced damage. Due to this effect, it preserves the urea and creatinine clearance function of these cells ultimately preventing acute renal failure.

ACKNOWLEDGMENTS

I would like to thank all the authors for contributing to the manuscript of the study.

CONFLICT OF INTEREST

We wish to declare that there are no known conflicts of interest with the research reported that could have influenced the outcome.

ETHICAL APPROVAL

The AEC (Animal Ethics Committee) Ziauddin University gave ethical review approval with protocol No: (2023-03/SS/FHS).

FUNDING

The project was self-funded.

AUTHORS CONTRIBUTION

SS: Acquisition of data, manuscript writing, compiling results, interpretation. AH: Revising critically and final approval of the article. ARB: Conception and design of the project. KJJB: Interpretation, analysis, critical review.

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