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ORIGINAL ARTICLE

Dl-3-n-Butylphthalide Inhibits Ferroptosis and Attenuates Sepsis-Associated Acute Kidney Injury by Activating the Nrf2/HO-1 Pathway

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SUMMARY

Background: **This study aimed to investigate the protective effects of Dl-3-n-butylphthalide (NBP) on sepsis-associated acute kidney injury (SA-AKI) and its underlying mechanisms.**
Methods: **A total of 32 mice were randomly divided into 4 groups (n = 8 per group): the control group (control group), lipopolysaccharide treatment group (LPS group), Dl-3-n-butylphthalide group (LPS + NBP group), and ferrostatin-1 group (LPS + Fer-1 group). Renal function was assessed by measuring serum creatinine (SCr) and blood urea nitrogen (BUN) levels. Oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and glutathione (GSH), were quantified in renal tissues. Histopathological evaluation of renal injury was performed using hematoxylin and eosin (HE) staining. The protein expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), solute carrier family 7 member 11 (SLC7A11), and glutathione peroxidase 4 (GPX4) were analyzed using immunohistochemistry and western blot.**
Results: **Compared with the control group, the LPS group exhibited significantly higher levels of SCr and BUN, decreased renal tissue levels of SOD, T-AOC, and GSH, as well as increased MDA levels and renal injury scores ($p < 0.05$). Furthermore, the LPS group exhibited significant downregulation of Nrf2, HO-1, GPX4, and SLC7A11 protein expression ($p < 0.05$). Compared with the LPS group, drug intervention significantly reversed the above-mentioned changes in both the LPS + NBP and LPS + Fer-1 groups ($p < 0.05$).**
Conclusions: **NBP can attenuate SA-AKI by activating the Nrf2/HO-1 pathway.**
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KEYWORDS

Dl-3-n-butylphthalide, ferroptosis, Nrf2/HO-1 pathway, acute kidney injury

INTRODUCTION

Acute kidney injury (AKI) is a highly prevalent renal disorder in clinical practice, characterized by a rapid increase in SCr levels, decreased urine output, or both occurring simultaneously [1]. Clinical epidemiological studies have shown that approximately 10 - 15% of hospital inpatients and 50% of intensive care unit (ICU) experience AKI [2]. The etiology of AKI is heterogeneous, commonly involving sepsis, renal ischemia/reperfusion injury, urinary tract obstruction, and nephrotoxic drugs [3]. Sepsis, a systemic inflammatory response syndrome triggered by infection, can lead to SA-AKI when it affects the kidneys. The pathophysiological mechanisms of SA-AKI are complex, and it is associated with high incidence and mortality rates [4-6]. However, effective therapeutic strategies for SA-AKI remain limited in clinical practice.

Ferroptosis is a novel form of cell death that was first proposed in 2012 [7]. Its main features include iron overload, depletion of GSH, and accumulation of lipid peroxides [8]. Emerging evidence suggests that SA-AKI is closely related to ferroptosis [9,10]. Inhibiting ferroptosis may serve as a promising strategy for alleviating renal injury.

NBP, a compound derived from the seeds of *Apium graveolens* Linn, was approved in 2002 for the prevention and treatment of ischemic stroke [11]. NBP exerts neuroprotective effects through multiple biological activities, including anti-inflammation, anti-oxidative stress, and anti-apoptosis, and is widely used in the treatment of neurological disorders [12,13]. Recent studies have found that NBP can attenuate renal injury by inhibiting inflammation and oxidative stress [14,15]. In the SA-AKI model, mice are usually exposed to adverse conditions such as inflammation and oxidative stress [16]. These findings suggest that NBP may serve as a potential therapeutic agent for SA-AKI.

In this study, we established an SA-AKI mouse model by intraperitoneal injection of LPS, and subsequently administered NBP and Fer-1 (a ferroptosis inhibitor) as interventions. The aim was to investigate the protective effects and underlying mechanisms of NBP in SA-AKI, thereby providing new insights and potential therapeutic strategies for the prevention and treatment of SA-AKI in clinical practice.

MATERIALS AND METHODS

Medicines and reagents

NBP (CSPC NBP Pharmaceutical Co., Ltd., Hebei, China); Fer-1 (Selleck, USA); LPS (Merck, USA); nuclear factor E2-related factor 2 (Nrf2) protein, solute carrier family 7 member 11 (SLC7A11), heme oxygenase-1 (HO-1), glutathione peroxidase 4 (GPX4), and β-actin (Affinity, Jiangsu, China); SCr and BUN detection kits (Jiancheng, Nanjing, China); MDA, SOD, T-AOC, and GSH detection kits (Beyotime, Jiangsu, China) were used.

Animals and treatment

The animal study was approved by the Laboratory Animal Management and Ethics Committee of Bengbu Medical University (approval number: 2025374). Male C57BL/6J mice, weighing 20 - 23 g and aged 7 - 8 weeks, were provided by Jiangsu Guxi Biotechnology Co., Ltd. After 7 days of adaptive feeding, 32 mice were randomly divided into 4 groups (n = 8 per group): Control group, LPS group, LPS + NBP group, and LPS + Fer-1 group. The SA-AKI model was established via intraperitoneal injection of LPS (10 mg/kg). In the LPS + NBP group, mice were administered an intraperitoneal injection of NBP solution (5 mg/kg/day) for 7 consecutive days before model establishment. Meanwhile, the other 3 groups received an equivalent volume of normal saline via the same route. For the LPS + Fer-1 group, ferrostatin-1 (10 mg/kg) was administered via intraperitoneal injection 30 minutes before LPS administration. Twelve hours after model establishment, mice were anesthetized with 1% pentobarbital sodium, and orbital venous blood and kidney tissues were collected. Mice were then euthanized. A portion of the kidneys was fixed in 4% paraformaldehyde for 24 hours and subsequently transported to the pathology department for histopathological analysis. The remaining kidneys were stored at -80°C for future biochemical assays.

Blood biochemical index analysis

The levels of SCr and BUN in mice were quantified using assay kits. All procedures were performed in strict compliance with the manufacturer's instructions.

Evaluation of oxidative stress

The levels of oxidative stress markers MDA, SOD, GSH, and T-AOC in renal tissues were determined. The absorbance values were measured respectively, in strict accordance with the kit instructions.

Renal pathology

Renal tissues were embedded in paraffin and sectioned into 4 μm-thick slices. Sections were stained with HE and imaged under a 400 x optical microscope. Renal injury was assessed by pathologists using Paller's scoring standard.

Immunohistochemistry (IHC)

Paraffin-embedded kidney sections (4 μm) were dewaxed, rehydrated, and repaired in EDTA buffer, followed by incubation in 3% H_2O_2 for 10 minutes. Sections were then blocked with 5% BSA for 45 minutes. The sections were incubated with the primary antibody at 4°C overnight, followed by incubation with the secondary antibody at 37°C for 1 hour. The sections were stained with 3,3'-diaminobenzidine and then counterstained with hematoxylin after observing the color development. The slices were fixed, dehydrated, and dried and then observed by a microscope. Ten randomly se-

lected fields of view from each section were used for evaluation and semi-quantitative analysis using Image J software (NIH, Bethesda, MD, USA).

Western blot

Renal tissue proteins were extracted using RIPA lysis buffer containing 1% PMSF. The protein samples were then separated through 10% SDS-PAGE and transferred to PVDF membranes afterward. The membranes were blocked for 30 minutes using a rapid blocking solution (Beyotime, Jiangsu, China) and then incubated overnight at 4°C with specific primary antibodies. On the following day, the membranes were incubated with secondary antibodies (Affinity) at room temperature for 2 hours. Finally, the target bands were visualized using a chemiluminescent imaging system (5200, Tanon, China) in conjunction with the Beyo ECL Plus kit (Beyotime Biotechnology) and quantified using Image J software.

Statistical analysis

All statistical analyses were conducted using SPSS 27.0 software (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA). Measurement data following normal distribution were presented as mean ± standard deviation (SD). The *t*-test and one-way ANOVA were applied to compare quantitative data between the groups. $p < 0.05$ was considered statistically significant. Data visualization and additional statistical analyses were performed using GraphPad Prism 10.1.2 software.

RESULTS

NBP improved renal function in mice

Compared with the control group, the LPS group exhibited significantly elevated levels of Scr and BUN ($p < 0.05$), confirming the successful induction of AKI by LPS. In comparison to the LPS group, both the LPS + NBP and LPS + Fer-1 groups showed markedly reduced levels of Scr and BUN (all $p < 0.05$) (Table 1), indicating that the drug interventions effectively mitigated LPS-induced renal injury. These findings demonstrate that NBP significantly alleviates LPS-induced acute kidney injury and improves renal function.

NBP ameliorated renal pathology in mice

The renal pathology examination of the control group revealed normal morphology, while the renal tissue from the LPS group exhibited significant pathological changes, including necrosis of tubular epithelial cells, loss of the brush border, and extensive infiltration of inflammatory cells ($p < 0.05$). Following the drug intervention, both the LPS + NBP group and the LPS + Fer-1 group showed significant improvement in renal pathological damage (all $p < 0.05$) (Figure 1). These results indicate that NBP effectively alleviates LPS-induced acute kidney injury.

NBP attenuated renal oxidative stress

Compared with the control group, the levels of SOD, T-AOC, and GSH in the kidneys of mice in the LPS group were markedly reduced, whereas the MDA level was significantly elevated ($p < 0.05$). In comparison to the LPS group, the LPS + NBP group exhibited increased levels of SOD, T-AOC, and GSH, along with a reduction in MDA levels ($p < 0.05$). NBP inhibited oxidative stress in the kidneys. Meanwhile, we found that Fer-1 also enhances the antioxidant capacity of the kidney ($p < 0.05$) (Table 2). This indicates that the renal protective effect of NBP may be related to ferroptosis.

NBP attenuated LPS-induced AKI by inhibiting ferroptosis

Compared with the control group, the LPS group showed significantly decreased expression levels of SLC 7A11 and GPX4 ($p < 0.05$). In contrast, both the LPS + NBP and LPS + Fer-1 groups exhibited significantly elevated expression levels of SLC7A11 and GPX4 compared to the LPS group (all $p < 0.05$) (Figure 2). The results indicate that NBP may alleviate SA-AKI by inhibiting ferroptosis.

NBP attenuated LPS-induced AKI via Nrf2/HO-1 pathway

Compared with the control group, the expression levels of Nrf2 and HO-1 in the LPS group were significantly reduced ($p < 0.05$). However, in the LPS + NBP group and the LPS + Fer-1 group, the expression levels of Nrf2 and HO-1 were significantly higher than those in the LPS group (all $p < 0.05$) (Figure 3). These results demonstrate that NBP may ameliorate SA-AKI by activating the Nrf2/HO-1 pathway.

DISCUSSION

This study confirmed the occurrence of ferroptosis in SA-AKI by evaluating renal function and ferroptosis-related markers in mice. NBP was found to significantly suppress ferroptosis, with its effects being comparable to Fer-1. Our previous research demonstrated that dexmedetomidine can attenuate total body radiation-induced acute liver injury in mice through the Nrf2/HO-1 pathway [17]. Some studies have also shown that activating the Nrf2/HO-1 pathway can inhibit ferroptosis [18,19]. This study found that NBP can inhibit ferroptosis and alleviate SA-AKI by upregulating the expression of Nrf2 and HO-1. These results suggest that NBP has great potential in the treatment of SA-AKI.

LPS, a key component of the cell wall in Gram-negative bacteria, plays a critical role in the pathogenesis of sepsis [20]. Consequently, LPS injection is widely used in animal studies to induce AKI models [21]. This study found that LPS could significantly increase the levels of SCr and BUN in mice and cause obvious pathological changes in the kidneys, which is consistent with our previous research results [22].

Table 1. The effect of NBP on SCr and BUN in mice.

Group	SCr (μmol/L)	BUN (mmol/L)
Control	11.73 ± 3.06	7.32 ± 2.56
LPS	49.88 ± 6.43 *	23.77 ± 2.85 *
LPS + NBP	30.14 ± 4.90 #	13.42 ± 3.13 #
LPS + Fer-1	24.60 ± 3.36 #	10.55 ± 1.72 #

Compared with the control group * $p < 0.05$, compared with the LPS group # $p < 0.05$.

Table 2. The effect of NBP on MDA, SOD, T-AOC, and GSH in mice.

Group	MDA (nmol/mg)	SOD (U/mg)	T-AOC (U/mg)	GSH (mg/L)
Control	7.23 ± 1.08	48.39 ± 4.70	2.30 ± 0.51	3.79 ± 0.33
LPS	16.90 ± 1.44 *	34.32 ± 3.26 *	0.72 ± 0.24 *	0.93 ± 0.18 *
LPS + NBP	10.40 ± 1.22 #	43.73 ± 2.56 #	1.49 ± 0.20 #	2.21 ± 0.25 #
LPS + Fer-1	12.11 ± 1.33 #	43.58 ± 3.26 #	1.49 ± 0.32 #	2.42 ± 0.48 #

Compared with the control group * $p < 0.05$, compared with the LPS group # $p < 0.05$.

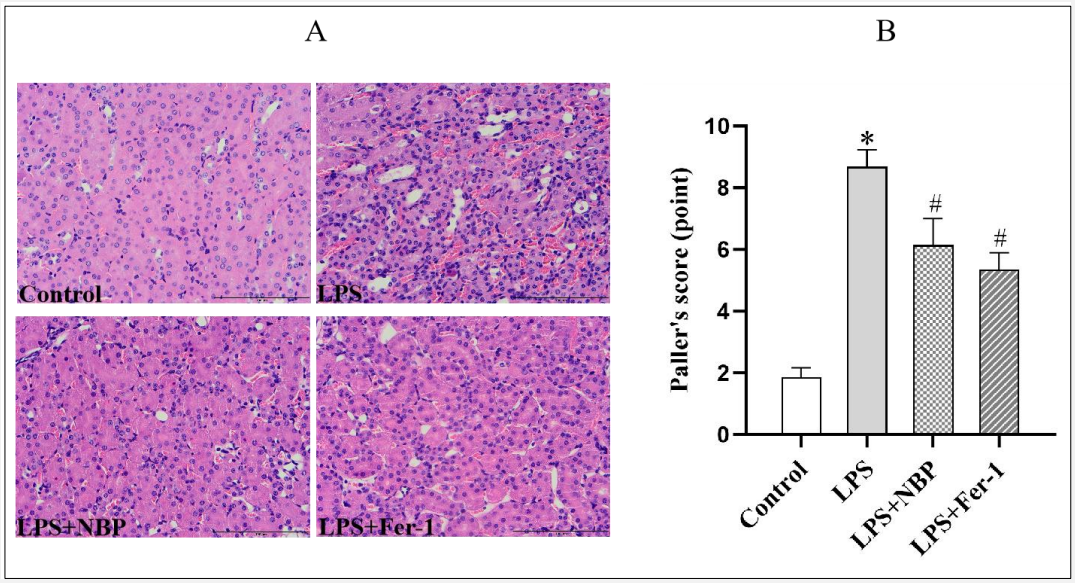

Figure 1. The pathological kidney changes in mice.

A Renal pathological changes under the light microscope (HE x 400), B pathological score of renal injury.
Compared with the control group * $p < 0.05$, compared with the LPS group # $p < 0.05$.

Oxidative stress is a key pathophysiological mechanism in SA-AKI and plays a critical role in its development and progression [23]. MDA, SOD, T-AOC, and GSH are commonly used as biomarkers to evaluate oxidative stress in the kidneys [24-26]. Previous studies have demonstrated that NBP possesses antioxidant proper-

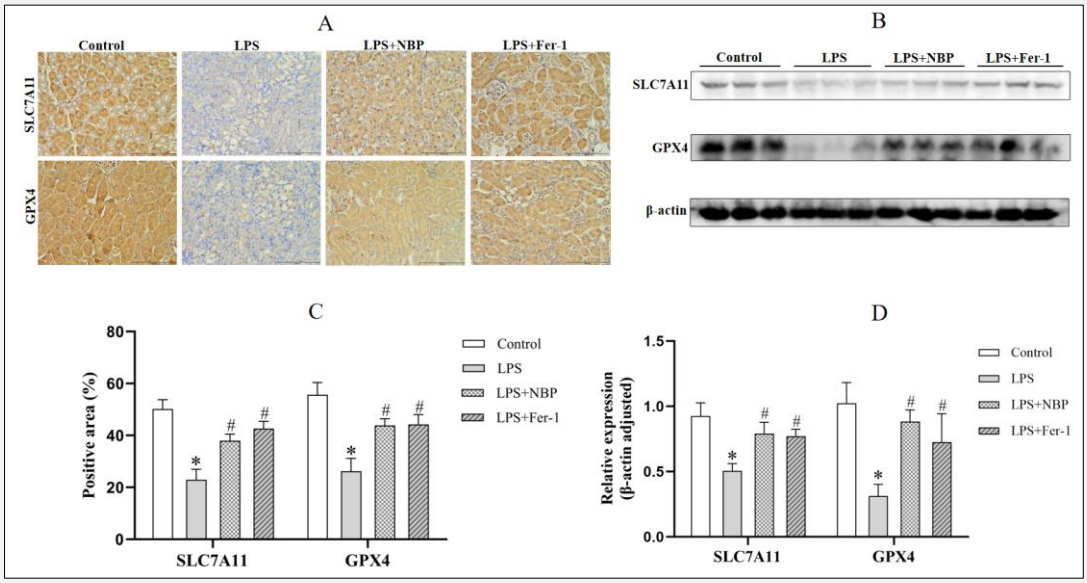

Figure 2. The effect of NBP on the expression of SLC7A11 and GPX4 in mice renal tissues.

A Immunohistochemical results of renal tissues (IHC x 400), B western blot band pattern, C statistical map of the immunohistochemical positive area, D chart of the statistical analysis of protein levels.
Compared with the control group * $p < 0.05$, compared with the LPS group # $p < 0.05$.

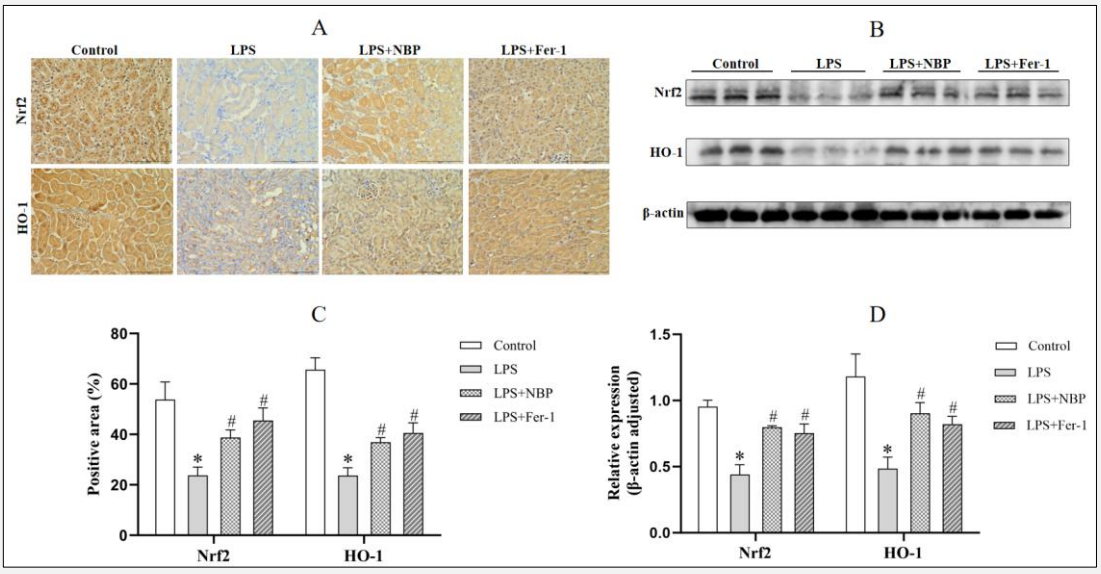

Figure 3. The effect of NBP on the expression of Nrf2 and HO-1 in mice renal tissues.

A Immunohistochemical results of renal tissues (IHC x 400), B western blot band pattern, C statistical map of the immunohistochemical positive area, D chart of the statistical analysis of protein levels.
Compared with the control group * $p < 0.05$, compared with the LPS group # $p < 0.05$.

ties, which is consistent with our results [27]. Therefore, we propose that NBP has the potential to mitigate LPS-induced oxidative stress injury in the kidneys.

Ferroptosis, a distinct form of regulated cell death, is closely associated with oxidative stress [28,29]. Recent studies have demonstrated that ferroptosis plays a critical role in SA-AKI, and inhibiting ferroptosis can significantly alleviate SA-AKI [30]. The cystine/glutamate antiporter system (System X_c^-) is composed of the light chain subunit (SLC7A11) and the heavy chain subunit (SLC3A2), and it can prevent ferroptosis by maintaining the level of GSH [9]. GSH is an essential cofactor for GPX4 to exert its antioxidant function [31]. GPX4 can reduce harmful lipid peroxides to harmless substances, thereby suppressing lipid peroxidation and ultimately inhibiting ferroptosis in cells, and it is considered one of the key markers of ferroptosis [32,33]. Inhibition of SLC7A11 results in GSH depletion and subsequent inactivation of GPX4, leading to lipid peroxidation-mediated ferroptosis [34]. Our research demonstrates that NBP and Fer-1 can significantly enhance the expression of SLC7A11 and GPX4 in the renal tissues of SA-AKI mice. These findings suggest that NBP may exert a protective effect on the kidneys by regulating ferroptosis.

Nrf2, a key transcription factor in the antioxidant response, regulates the expression of various antioxidant enzymes, including HO-1 [35]. HO-1 exhibits both anti-inflammatory and antioxidant properties, effectively suppressing intracellular ROS accumulation and mitigating oxidative stress-induced damage [36]. The Nrf2/HO-1 signaling pathway plays a critical role in inhibiting lipid peroxidation and ferroptosis, thereby exerting protective effects against SA-AKI [37,38]. Consequently, elucidating the mechanisms of this pathway holds significant promise for the clinical management of SA-AKI. NBP could inhibit ferroptosis and ameliorate brain injury by activating the Nrf2/HO-1 pathway [39]. In our study, LPS treatment suppressed the Nrf2/HO-1 pathway, whereas NBP and Fer-1 restored its activity. These results indicate that NBP may exert renal protective effects through the activation of the Nrf2/HO-1 pathway. However, this study has certain limitations. First, the research was conducted solely in an LPS-induced AKI mouse model, and future studies will explore other acute kidney injury models to validate these findings. Second, while this study primarily focused on the Nrf2/HO-1 pathway, additional potential mechanisms still need to be further explored.

CONCLUSION

In conclusion, this study demonstrated that NBP can inhibit ferroptosis and alleviate SA-AKI by activating the Nrf2/HO-1 pathway. This discovery provides a new approach to the treatment of SA-AKI. Future studies will focus on further elucidating the clinical application value of NBP and exploring its underlying mechanisms in greater depth.

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Ethical Approval Statement:

The animal study was reviewed and approved by the Animal Ethics Committee of Bengbu Medical University (approval number: 2025374).

Declaration of Interest:

No potential conflicts of interest concerning this article were declared.

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