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Original Article

Quercetin Ameliorates Sepsis-Related Acute Kidney Injury through Inhibition of Ferroptosis in Renal Tubular Epithelial Cells via the Nrf-2/GPX4 Pathway

Bo Long[1,#], Liangchen Li[2,#], Xiaoqiang Liu[2,*]

[1]Department of Critical Care Medicine, People's Hospital of Chongqing Hechuan, Chongqing, CHINA.
[2]Department of Emergency, The People' s Hospital of Nanchuan, Chongqing, CHINA.
[#]These authors contributed equally to this work as co-first authors

ABSTRACT

Objectives: This study evaluates the therapeutic effect of quercetin on Sepsis-related Acute Kidney Injury (S-AKI) and explores its potential mechanisms to provide theoretical support for its use in treating S-AKI. **Materials and Methods:** In this study, approximately 40 mice were assigned to 4 groups: Mock, Lipopolysaccharide (LPS), and two quercetin dosage groups (low: 25 mg/kg and high: 50 mg/kg doses). All groups except Mock were administered 5 mg/kg LPS intraperitoneally to establish a model of S-AKI. Renal tissue morphological features were examined *via* Hematoxylin and Eosin (H&E) staining and histopathological scoring. Serum levels of the renal function markers Creatinine (Cr), Blood Urea Nitrogen (BUN), and inflammatory factors IL-1β, TNF-α, and IL-6 were measured. A septic injury model was developed in HK-2 cells using LPS stimulation, and iron content in HK-2 cells and mouse renal tissues was measured. The expressions of ferroptosis-related genes (*FPN-1*, *COX-2*, *GPX4*, and *ACSL4*) were evaluated *via* RT-PCR analysis. Spectrophotometry measured Malondialdehyde (MDA) and Glutathione (GSH) levels in HK-2 cells and mouse renal tissues. ELISA was used to quantify Nrf-2 and GPX4 protein levels. The effects of silencing Nrf-2 or GPX4 in HK-2 cells using shRNA were evaluated by measuring MDA and GSH levels. **Results:** In contrast to the LPS group, quercetin intervention (both doses) remarkably improved the morphology of mouse renal tissues, markedly reduced pathological scores of kidney injury ($p < 0.05$), lowered serum levels of inflammatory mediators ($p < 0.05$), and decreased levels of kidney function markers Cr and BUN ($p < 0.05$), showing a dose-dependent effect. Quercetin substantially reduced the content of ferrous ions and the expression of ferroptosis-promoting genes *ACSL4* and *COX-2* in HK-2 cells and mouse kidney tissues ($p < 0.05$), while upregulating the level of ferroptosis-inhibiting genes *GPX4* and *FPN-1* ($p < 0.05$), decreasing MDA content and increasing GSH levels ($p < 0.05$). The levels of Nrf-2 and GPX4 in HK-2 cells and mouse kidney tissues induced by LPS were significantly downregulated and were reversed under quercetin intervention ($p < 0.001$). Silencing of the Nrf-2 or GPX4 genes could antagonize the protective effects of quercetin on MDA increase and GSH reduction in HK-2 cells ($p < 0.05$). **Conclusion:** Quercetin can alleviate S-AKI by suppressing ferroptosis in renal tubular epithelial cells, with its mechanism likely involving the stimulation of the Nrf-2/GPX4 pathway.

Keywords: Sepsis, Acute kidney injury, Ferroptosis, Quercetin, Nrf-2, GPX4.

Correspondence:
Dr. Xiaoqiang Liu
Department of Emergency, The People' s Hospital of Nanchuan, 4-22-3, Jinkeshi Jincheng, Xicheng Street Office, Nanchuan District, Chongqing City-401520, CHINA.
Email: tempttwo@163.com

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INTRODUCTION

Sepsis, a systemic inflammatory reaction syndrome, is often triggered by infection, which can lead to multi-organ failure in severe cases. The prominent clinical outcome of sepsis is sepsis-associated Acute Kidney Injury (S-AKI), which greatly affects patient prognosis. Clinical statistics indicate that in intensive care units, about 50% of AKI cases are caused by sepsis, with an associated mortality rate of up to 60%.[1] Ferroptosis is a type of Programmed Cell Death (PCD) associated with Oxidative Stress (OS) and elevated iron levels that has recently been discovered as an important pathogenic process in S-AKI.[2-4] During this process, iron accumulation and catalytic action lead to Polyunsaturated Fatty Acids (PUFAs) peroxidation. The cell's antioxidant defence system, which includes Glutathione (GSH) and GSH peroxidase, is compromised and unable to efficiently remove the products of peroxidation, ultimately resulting in cell death and the breakdown of the structure and function of the cell membrane.[5,6]

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Naturally occurring flavonoid compound, quercetin, is abundantly present in fruits, vegetables, and various natural medicinal plants commonly included in our daily diets.[7] Structurally, quercetin molecules contain a catechol group and a hydroxyl group at C3, which confer strong reductive properties, and it has been demonstrated to have significant antioxidant activity in pharmacological studies.[8] Research has shown that quercetin can alleviate cortical inflammation and OS caused by traumatic brain injury by triggering the Nrf2/HO-1 pathway.[9] It can also inhibit the production of TNF-α in macrophages, playing an anti-inflammatory role by suppressing various inflammatory pathways.[10] Moreover, GSH Peroxidase 4 (GPX4) is an essential antioxidant enzyme that prevents ferroptosis. However, the mechanisms by which quercetin utilizes the Nrf-2/GPX4 pathway to inhibit ferroptosis in Epithelial Cells (ECs) of the renal tubule, thereby ameliorating S-AKI, remain unclear.

This study utilizes LPS stimulation to establish a mouse model of S-AKI and an HK-2 cell septic injury model to examine the preventive impacts of quercetin on renal cell damage and to examine its specific mechanisms of action. The goal was to provide a theoretical basis for applying quercetin to treat S-AKI.

MATERIALS AND METHODS

Cells and Reagents

The HK-2 human renal tubular EC line was acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were allowed to grow in Dulbecco's Modified Eagle Medium (DMEM; Gibco), with 10% Fetal Bovine Serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), at 37 °C and 5% CO_2. HK-2 cells were treated for 16 h with 10 μg/mL LPS to simulate S-AKI. The experimental groups included a Mock, an LPS, a low-dose quercetin (25 μmol/L), a high-dose quercetin (50 μmol/L), and a Ferrostatin-1 (2 μmol/L) group.[11,12] Except for the Mock group, all groups were cultured in a medium containing LPS (10 μg/mL) and treated with the respective concentrations of compounds.

Ferrostatin-1 (Fer-1; HY-100579; MedChemExpress, Inc.); LPS (HY-D1056; MedChemExpress, Inc.); Quercetin (HY-18085; MCE; MedChemExpress, Inc.); ELISA kits for IL-β, IL-6, and TNF-α (Wuhan Kelu Biological Technology Co., Ltd.); Nrf-2 and GPX4 ELISA kits (ABclonal, ABclonal Biotech Co., Ltd., Shanghai).

Animal Grouping and Development of the S-AKI Mouse Model

The present study developed an S-AKI mouse model using an Intraperitoneal injection (IP) of LPS. For the *in vivo* study, 5-week-old SPF-grade BALB/c mice were assigned to four groups (n = 10) (18-22 g) and administered the following treatments: (1) Mock group: Each mouse was administered a 0.2 mL saline injection intraperitoneally. (2) LPS group: Each mouse received an IP injection of an isotonic solution of LPS (dose of 15 mg/kg). (3) Low-dose group: Mice were pre-treated with 25 mg/kg of quercetin through oral gavage for 3 days before the experiment. On the first day of the experiment, 1 hr after quercetin administration, they received an intraperitoneally LPS injection (15 mg/kg). (4) High-dose group: Mice were pre-treated with 50 mg/kg of quercetin *via* oral gavage for 3 days before the experiment. On the first day of the experiment, 1 hr after quercetin administration, they received an intraperitoneally LPS injection (15 mg/kg).

Mice in each group were euthanized after 24 hr of LPS injection, and samples of blood and renal tissues were collected for analysis of relevant indicators.

Renal Function Testing

Mice were humanely euthanized, and blood was collected from the aorta. The blood samples were kept for 20 min at 25°C, and then the serum was separated by spinning for 20 min at 2000 rpm. Serum levels of BUN and Creatinine (Cr) were quantified using a Roche automatic biochemical analyzer. The experiment was repeated thrice to validate it.

Quantification of Serum Concentrations of IL-1β, TNF-α, and IL-6 *via* ELISA

The blood sample was collected, and the serum was separated as described in the previous section. The serum levels of IL-1β, TNF-α, and IL-6 were quantified using ELISA according to the protocols provided with the kits. The experiment was replicated thrice.

Histopathological Analysis

Mouse kidney tissues were kept in 10% formalin for fixation, followed by the paraffin embedding. Harvested tissue (3 μm thickness) sections were stained with H&E for histopathological analysis. Three independent physicians who were not involved in the experiment blindly analyzed and scored the renal pathological damage. Specific renal tubular damage included tubular EC proliferation, vacuolar degeneration, loss of brush border, tubular cast formation, and EC sloughing off. The scoring criteria for AKI were as follows: 0 points for normal kidney tissue; 1 point for <25% of tubular damage; 2 points for 25-50% tubular damage; 3 points for 50-75% tubular damage; and 4 points for >75% tubular damage.[13] The experiment was repeated thrice for validation.

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 (Gibco; Thermo Fisher Scientific, Inc.) analyzed cell viability or survival undergoing ferroptosis. HK-2 cells (1×10^6 cells/mL) were grown in a 96-well plate followed by 12 hr incubation. After that, cells were treated with LPS (10 μg/mL) and quercetin (25 μmol/mL or 50 μmol/mL) or Ferrostatin-1 (2 μmol/mL) for 24 hr. After treatment, 10 μL of CCK-8 solution

was added to each well, and kept the plate at 37°C in a 5% CO_2 incubator for 2 hr. A microplate reader assessed cell viability by observing the absorbance (OD) at 450 nm. Each experiment was repeated thrice.

MTT Assay

For the MTT assay, HK-2 cells (1×10^6 cells/mL) were again grown in a 96-well plate followed by 12 hr of incubation. Post-incubation, cells were exposed to LPS (10 µg/mL) and quercetin (both doses). At 0, 12, 24, 36, and 48 hr after treatment, 10 µL of MTT dye (Thermo Fisher Scientific, Rockford, IL, USA) (5 mg/mL in PBS) was added to each well. After that, the plates were kept for 3 hr at 37°C in the dark. To dissolve the formazan crystals, DMSO (100 µL) was added to the media after incubation. The OD was measured at 570 nm using a multi-mode microplate reader (Molecular Devices, Sunnyvale, USA). A cell survival curve was plotted, and cell viability was determined. Each experiment was repeated in triplicates.

RT-PCR Assay

RNA was isolated using an RNA extraction kit as per the manufacturer's recommendations. Moreover, qRT-PCR evaluated mRNA expression levels for *GPX4, Nrf-2, ACSL4, COX-2*, and *FPN1*. Each experiment was repeated in triplicates. List of primer sequences used in this study:

Name of gene	Primer sequence（5′-3′）
GPX4	Forward: AGTGGTGTAAGAACGGCTGCAAG
	Reverse: TAGGCACGGCAGGTAGATACCTTC
Nrf-2	Forward: CCGGGAAATTGCGGAACTGTGTAG
	Reverse: TTCCAAGGTAGAAGGCACCCTTC
ACSL4	Forward: TGTTATGAGCAAGGTTCAAGAGATG
	Reverse: AGATTACAAAGAGGGGCGTCA
COX-2	Forward: TGGCCATTCCCATTTAGAGAGTA
	Reverse: CTTGCTTCCTGCTCCCATGTATG
FPN-1	Forward: GACACTCTCTCGAATGAGGAGAT
	Reverse: ACTCTTTGTGTTCCTCGGTCTTG
GAPDH	Forward: 5'-GAAGGTGAAGGTCGGAGTCAAC-3'
	Reverse: 5'-CAGAGTTAAAAGCAGCCCTGGT-3'

Detection of Divalent Iron Ions

Approximately 5×10^5 cells from each group were collected by discarding the culture medium and digesting with trypsin, followed by low-speed centrifugation to gather cells in an EP tube. After washing the cells (twice) with precooled PBS buffer, 200 µL of cell lysis solution provided by the iron ion assay kit (colorimetric method) was added for resuspension. The assay was conducted in line with the manufacturer's protocols. The OD was obtained at 550 nm by an ELISA reader. The readings were taken in triplicates.

Lipid Peroxidation Indicators Malondialdehyde (MDA) and GSH Content

The content of MDA in cell homogenates was analyzed by measuring the absorbance of the reaction product of thiobarbituric acid and MDA (thiobarbituric acid reactive substances, TBARS) at 535 nm using spectrophotometry. The content of GSH in cell homogenates was quantified at 412 nm using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The experiment was repeated three times.

Lentiviral Mediated Knockdown of Nrf-2 and GPX4 in HK-2 Cells

To downregulate the level of *Nrf-2* and *GPX4* in HK-2 cells, lentiviral infection was used, following these specific steps: (1) Cell Preparation: 18-24 hours before lentiviral transfection, HK-2 cells were seeded in a 24-well plate with a density of 1×10^5 cells per well, ensuring cell density was about 2×10^5 cells/well at the time of transfection. (2) Infection: 2 mL of fresh media with 6 µg/mL polybrene was added to replace the original medium, and an appropriate quantity of viral suspension was introduced. (3) Post-infection Medium Change: After 24 hr of infection, the medium containing the virus was replaced with a fresh medium. (4) Further Cultivation: The cells were further grown for 48-72 hr until the lentivirus containing fluorescent protein was clearly visible under a fluorescence microscope. Fluorescence-Activated Cell Sorting (FACS) was used to check the transfection efficiency before proceeding with further experimental procedures. The experiment was repeated thrice.

Statistical Analysis

Data was statistically evaluated *via* GraphPad Prism 7.0 software, Inc. and illustrated as Mean ± Standard Deviation (SD). An independent samples *t*-test was used to compare the two groups. *p*-values < 0.05 were regarded as statistically significant.

RESULTS

Quercetin Alleviates LPS-Mediated S-AKI and Inflammatory Responses

In contrast to the Mock group, the LPS group showed remarkably higher Cr and BUN serum levels (Figure 1A-1B, Table 1, $p < 0.001$), indicating the successful establishment of an S-AKI mouse model induced by LPS. Further, ELISA results revealed that inflammatory factors were substantially enhanced in the serum of the LPS group relative to the Mock group (Figure 1C-1E, Table 1, $p < 0.001$).

In comparison to the LPS group, intervention with quercetin (25 mg/kg or 50 mg/kg) significantly reduced the levels of renal function indicators Cr ($p < 0.01$) and BUN ($p < 0.001$) in the serum of S-AKI mice (Figure 1A-1B, Table 1), and also decreased the levels of IL-1β ($p<0.01$), TNF-α ($p < 0.001$), and IL-6 ($p < 0.001$) (Figure 1C-1E, Table 1), showing a dose-dependent effect. Consistent with this trend, H&E staining results depicted that under the intervention of both quercetin doses, the LPS group's injury scores were significantly reduced (Figure 1F). There was a marked alleviation in renal tissue damage, including reduced tissue edema, fewer necrotic patches in the tubules, and significantly lower levels of inflammatory infiltration (Figure 1G).

Quercetin Mitigates LPS-Mediated Reduction in HK-2 Cell Viability

The protective effects of quercetin were quantified through MTT assays, and cell survival curves were plotted (Figure 2A). In contrast to the LPS group, quercetin intervention (both doses) alleviated the decrease in HK-2 cell viability induced by LPS ($p < 0.001$). Notably, 50 μmol/L quercetin substantially promoted the recovery of HK-2 cell activity at 36 hr and 48 hr time points. Furthermore, CCK-8 assays demonstrated that quercetin intervention (both doses) considerably reduced LPS-mediated HK-2 cell death ($p < 0.001$), with a clear dose-dependent effect (Figure 2B). Additionally, the intervention with the ferroptosis inhibitor Fer-1 (2 μmol/L) reversed the LPS-mediated cell death in HK-2 cells (Figure 2B), suggesting that the HK-2 cell injury model is related to cellular ferroptosis. The protective mechanism of quercetin on HK-2 cells may involve the inhibition of ferroptosis in these cells.

Quercetin Inhibits LPS-Induced Ferroptosis in HK-2 Cells

To confirm whether the protective effects of quercetin on HK-2 cells are related to its ability to suppress ferroptosis, we measured the intracellular divalent iron ions (Fe^{2+}). Compared to the Mock group, LPS induction led to an accumulation of Fe^{2+} ions in HK-2 cells, while quercetin intervention significantly inhibited the abnormal increase in iron ion content within the cells (Table 2 and Figure 3A). We further examined the levels of

Table 1: Levels of Cr, BUN and IL-1β, TNF-α and IL-6 in the serum of AKI mouse model ($\bar{x}$±s).

Indicators	Mock group	LPS group	Low-dose group（25 mg/kg）	High-dose group（50 mg/kg）
Cr (mol/L)	16.23±2.93	132.18±22.77$^{###}$	103.29±18.82**	57.51±7.77***
BUN (mmol/L)	6.01±0.63	30.71±8.44$^{###}$	24.19±4.17**	17.24±3.93***
IL-1β (pg/mL)	20.90±5.55	113.98±22.85$^{###}$	86.93±8.25**	46.33±13.66**
TNF-α(pg/mL)	21.38±5.33	108.97±20.00$^{###}$	66.04±15.86**	35.99±12.11***
IL-6 (pg/mL)	22.90±5.68	64.02±15.86$^{###}$	47.00±6.56***	25.50±10.00***

AKI, acute kidney injury; LPS, lipopolysaccharide; Cr, creatinine; BUN, blood urea nitrogen; IL, interleukin; TNF-α; Tumor Necrosis Factor-α. Relative to the Mock group, ### $p < 0.001$. relative to the LPS group, $^{**}p < 0.01$, $^{***}p < 0.001$.

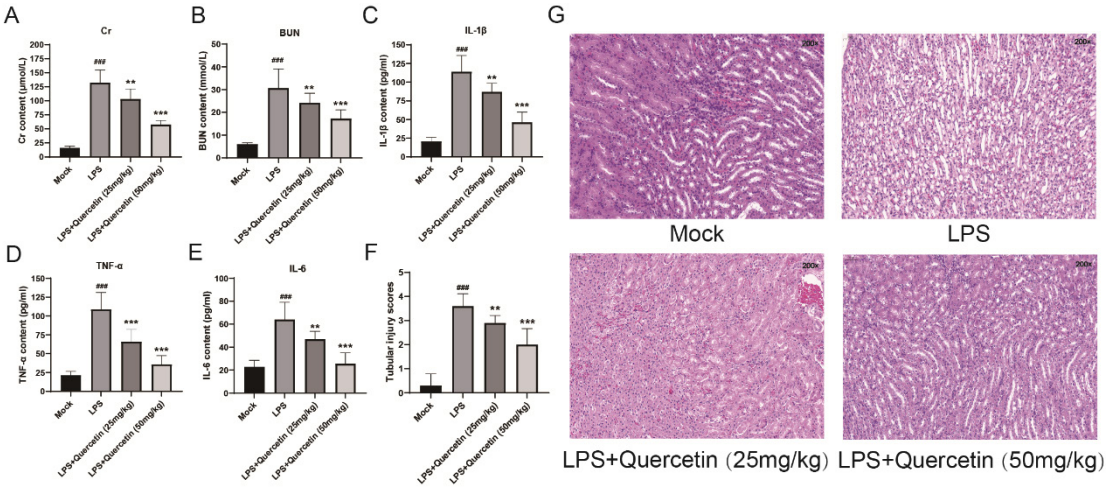

Figure 1: Quercetin's Protective Effects on Renal Injury in Mice with S-AKI. (A-B) An automatic biochemical analyzer determined levels of Cr and BUN in mouse blood. (C-E) levels of IL-1β, TNF-α, and IL-6 in mouse serum quantified by ELISA. (F) Histological analysis of tubular injury scoring in the mouse model. (G) H&E staining is used to observe mouse kidney tissue morphology (200x magnification). S-AKI, sepsis-related acute kidney injury; LPS, lipopolysaccharide; Cr, creatinine; BUN, blood urea nitrogen; IL, interleukin; TNF-α; tumour necrosis factor-α; ELISA, enzyme linked immunosorbent assay. In contrast to the Mock group, ### $p < 0.001$. Relative to the LPS group, $^{**}p < 0.01$, $^{***}p < 0.001$.

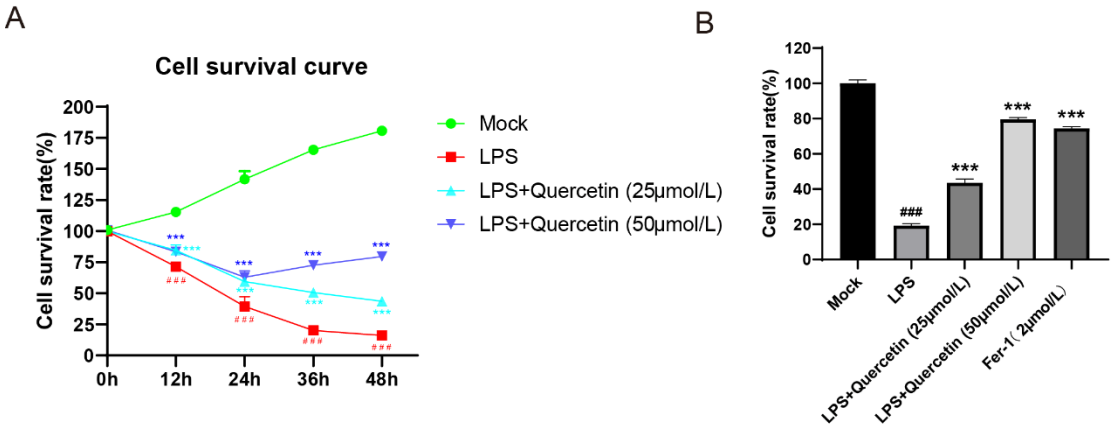

Figure 2: Impact of Quercetin on LPS-Mediated HK-2 Cell Viability. (A) Cell viability of HK-2 cells quantified using the MTT assay. (B) Quantification of ferroptosis in HK-2 cells using the CCK-8 assay. LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CCK-8, Cell Counting Kit-8. In contrast to the Mock group, ### $p < 0.001$. Relative to the LPS group, ***$p < 0.001$.

gene expression related to ferroptosis (*FPN-1, GPX4, COX-2*, and *ACSL-4*). Results showed that under LPS induction, the mRNA expression levels of ferroptosis-inhibiting genes FPN-1 and GPX4 were significantly downregulated (Figure 3B-3C), whereas the levels of ferroptosis-inducing genes *COX-2* and *ACSL-4* increased (Figure 3D-3E). Under the intervention with quercetin (both doses), the levels of *FPN-1* and *GPX4* in HK-2 cells were markedly upregulated (Figure 3B-3C), and the expression of *COX-2* and *ACSL-4* was reduced (Figure 3D-3E).

Lipid peroxidation's product, MDA, is frequently used to detect oxidative damage in ferroptosis. Experimental results showed that the MDA content in HK-2 cells from the LPS group was substantially increased than that in the Mock group, but the intervention with quercetin and the ferroptosis inhibitor Fer-1 remarkably reduced the MDA content in the HK-2 cell injury model (Table 2 and Figure 3F). Moreover, reduced GSH, a crucial intracellular antioxidant, was markedly lower in the LPS group than in the Mock group. The intervention with quercetin and Fer-1 significantly promoted the recovery of GSH levels in the HK-2 cell injury model (Table 2 and Figure 3G). Overall, the intervention with quercetin inhibited LPS-stimulated ferroptosis in HK-2 cells.

Quercetin Inhibits Ferroptosis *via* the Nrf-2/GPX4 Signaling Pathway

Nrf-2 is a major modulator of the antioxidant response, involved in regulating cellular iron metabolism and protecting cells from oxidative damage and ferroptosis. Its downstream antioxidant gene, GPX4, is a key regulatory enzyme that scavenges lipid peroxides within cells. This study used an ELISA assay to measure the expression of Nrf-2 and GPX4 in HK-2 cells and sepsis-induced AKI mouse kidney tissues. In comparison to the Mock group, a substantial reduction in the protein expression of Nrf-2 and GPX4 was observed in HK-2 cells induced by LPS (Figure 4A-4B) and in renal tissues of sepsis-induced AKI mice (Figure 4C-4D) ($p < 0.001$). However, the intervention or treatment with quercetin remarkably increased the expression levels of Nrf-2 and GPX4 ($p < 0.001$), depicting that Nrf-2 and GPX4 may be involved in the regulatory process by which quercetin inhibits cellular ferroptosis.

To further confirm whether quercetin could upregulate these factors, HK-2 cells were subjected to gene silencing of Nrf-2 and GPX4 using shRNA (Figure 4E-4F). The effects of quercetin on MDA and GSH levels in HK-2 cells were then assessed. The results showed that silencing either Nrf-2 or GPX4 led to an accumulation of MDA and a reduction in GSH levels in HK-2 cells. When Nrf-2 or GPX4 was silenced, quercetin did not reduce the accumulation of MDA nor increase the levels of GSH in HK-2 cells (Figure 4G-4H). This indicates that Nrf-2 and GPX4 regulate the inhibitory effect of quercetin on cellular ferroptosis.

DISCUSSION

This study was designed to evaluate the protective role of quercetin on S-AKI and to determine if this protection is directly related to the mitigation of the inflammatory response. To this end, we initially established a sepsis-induced AKI mouse model through LPS stimulation. This model, by mimicking the inflammatory environment of sepsis, provided an ideal platform to assess the efficacy of quercetin. Our experimental results demonstrate that quercetin significantly protects renal cells and effectively reduces the inflammation caused by LPS. However, these findings have not yet clarified whether quercetin directly counteracts renal tissue damage. To delve deeper into this question, we further constructed an injury model using HK-2 cells and tested various concentrations of quercetin (25 μmol/L and 50 μmol/L). We observed that quercetin intervention significantly promoted the growth of HK-2 cells damaged by sepsis and reduced cell mortality. These results showed that the protective effects of quercetin extend beyond merely alleviating the inflammatory response; it may also directly protect renal tissues from damage.

Table 2: Fe2+, MDA and GSH content levels in HK-2 cells（x̄±s).

Indicators	Mock group	LPS group	Low-dose group（25 mg/kg）	High-dose group（50 mg/kg）	Fer-1 group（2 μmol/L）
Fe^{2+}（nM）	20.08±0.46	41.47±1.96$^{###}$	34.12±0.86*	24.90±0.88**	28.21±0.50**
MDA（nmol/mg prot）	0.52±0.05	2.71±0.13$^{###}$	1.89±0.09***	0.98±0.08***	0.87±0.11***
GSH（mol/g prot）	44.69±1.00	18.67±1.05$^{###}$	23.63±1.16***	35.79±0.45***	36.31±0.73***

LPS, lipopolysaccharide; MDA, malondialdehyde; GSH, Glutathione. In comparison to the Mock group, ### P<0.001; relative to LPS group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

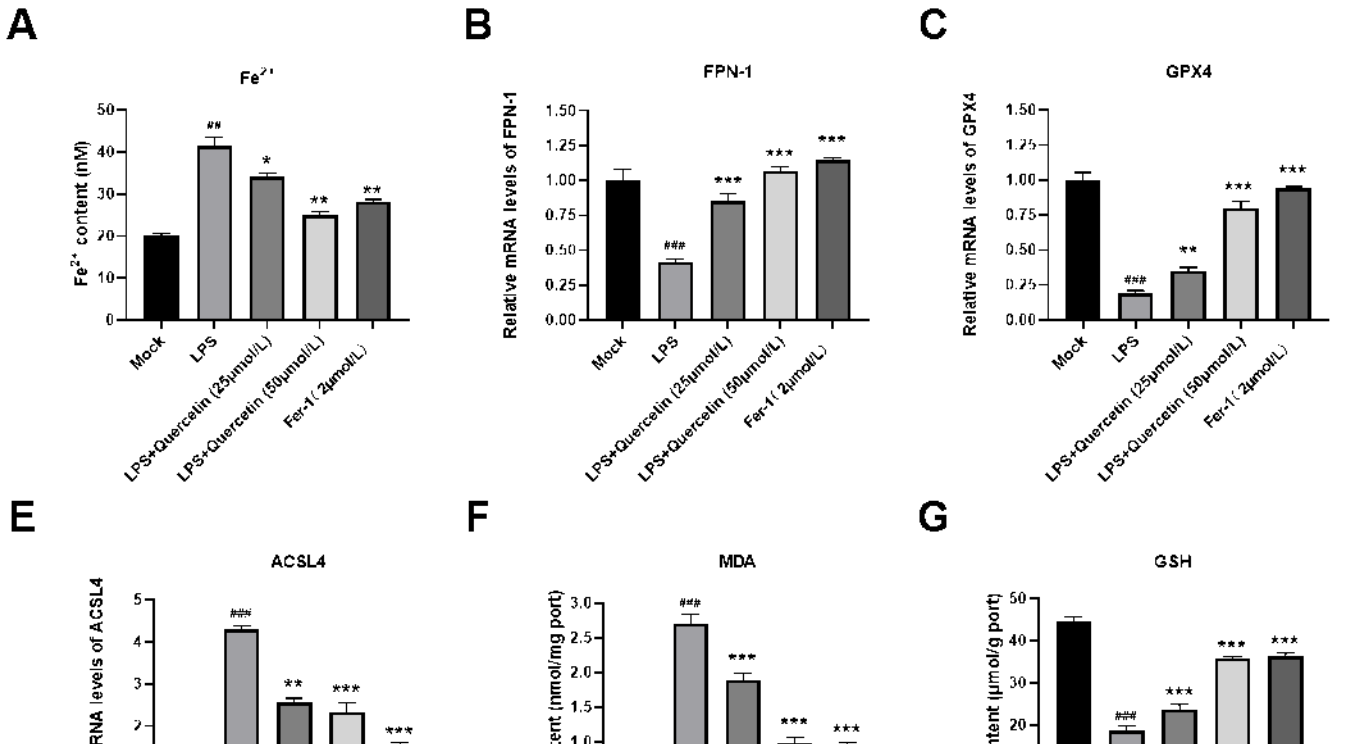

Figure 3: Quercetin's Inhibition of Ferroptosis in HK-2 Cells. (A) Measurement of Fe^{2+} content in HK-2 cells using a reagent kit. (B-E) RT-PCR analysis of mRNA levels of ferroptosis-related genes *FPN-1*, *GPX4*, *COX-2*, and *ACSL-4* in HK-2 cells. (F) Spectrophotometric determination of MDA content in HK-2 cells. (G) Measurement of reduced GSH level in HK-2 cells using a glutathione assay kit. LPS, lipopolysaccharide; MDA, malondialdehyde; GSH, Glutathione. In contrast to the Mock group, ### $p < 0.001$; relative to the LPS group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Current research indicates that ferroptosis of renal tubular cells is a significant mechanism in kidney injury diseases.[11,12] Similarly, our study found that the ferroptosis inhibitor Ferrostatin-1 (2 μmol/L) significantly alleviated LPS-stimulated damage in HK-2 cells. Therefore, we investigate whether the specific mechanisms underlying the LPS-stimulated S-AKI mouse model and HK-2 cell injury model are related to ferroptosis in renal tubular cells. Moreover, we hypothesize that the therapeutic action of quercetin in treating septic damage may involve inhibiting the ferroptosis process in renal tubular cells.

Ferroptosis is a type of PCD that relies on iron. It differs from apoptosis, autophagy, and necrosis in terms of its shape, genetic makeup, metabolism, and molecular biology. The mechanisms primarily involve abnormal cellular iron metabolism, lipid peroxidation of membranes, and the loss of antioxidant system functions.[13] The pathological accumulation of iron within cells is a key step in the onset of ferroptosis. Currently, FPN1 is the only identified iron-exporting protein that can mediate the expulsion of excess iron ions from cells. Under conditions like infection, inflammation, tumors, and drug stimuli, abnormal cellular iron metabolism mediated by FPN-1 can lead to pathological iron accumulation within cells.[14] Moreover, the loss of antioxidant system functions and excessive lipid peroxidation also play crucial roles in promoting ferroptosis. The GSH system is the main antioxidant system within cells, where the core enzyme GPX4 uses GSH as a substrate to impede the membrane phospholipid hydroperoxides to nontoxic alcohols, thereby reducing the Reactive Oxygen Species (ROS) formation. Inhibition of GPX4

results in the oxidation of PUFAs and the production of MDA, a lipid peroxidation product.[15,16] ACSL4 and Lysophosphatidyl choline Acyltransferase 3 (LPCAT3) promote ferroptosis by incorporating PUFAs and MDA into cellular phospholipids.[17-19] During inflammation or infection-mediated cellular ferroptosis, COX-2 is considered a critical marker involved in the cellular OS process. Elevated expression or activity levels of COX-2 can lead to overproduction of ROS, promoting the process of ferroptosis. COX-2 is a significant inflammatory signaling molecule involved in inflammation-mediated apoptosis and other processes.[20,21] Thus, cellular ferrous ions, iron metabolism gene FPN-1, antioxidant factor GSH, lipid peroxidation product MDA, and ferroptosis-related genes (*COX-2, ACSL4*, and *GPX4*) are all important indicators for assessing cellular ferroptosis.

In the current study, we first observed a remarkable increase in the content of divalent iron ions in both the kidney tissues of LPS-stimulated S-AKI mice and HK-2 cells. Concurrently, a substantial downregulation was observed in the level of the *FPN-1* gene. We speculate that the inflammatory response mediated by LPS or LPS itself inhibits FPN-1-mediated iron ion metabolism, leading to the excessive incorporation of ferrous ions within cells and initiating the process of ferroptosis. After that, we detected an increased level of *COX-2* and *ACSL4* and a reduced level of *GPX4* and the content of GSH in both the HK-2 cell injury model and AKI mouse kidney tissues, indicating a loss of cellular antioxidant protective function. Finally, a significant MDA content increase within cells or tissues suggested lipid peroxidation. Furthermore, intervention with the inhibitor Ferrostatin-1 considerably reversed the expression imbalance of ferroptosis-related genes. These experimental results all indicate that ferroptosis and oxidative stress are key factors in LPS-induced septic AKI. Therefore, we further explored whether quercetin could regulate the ferroptosis process in renal tubular cells induced by LPS. The results were consistent with our hypothesis: regarding cellular iron metabolism, quercetin intervention significantly upregulated the level of FPN-1 in HK-2 cells or AKI mouse kidney tissues. It reduced the excessive iron accumulation in renal cells. Regarding lipid oxidation or OS, quercetin intervention was able to upregulate the level of *GPX4* and increase the intracellular content of reduced GSH while also inhibiting the expression of *COX-2* and *ACSL4* genes. This helps to reduce the production of cellular ROS and the excessive accumulation of MDA. Overall, our study results indicate that quercetin can inhibit the ferroptosis process mediated by LPS in cells.

The nuclear transcription factor Nrf2 is a major antioxidant response regulator and can be induced to participate in intracellular iron metabolism and iron concentration regulation, protecting cells from oxidative damage and ferroptosis.[22-24] GPX4, a selenoprotein in mammals that repairs lipid peroxidation damage, is an important protein in the Nrf2 transcription pathway and a crucial inhibitor of ferroptosis.[25] Knocking out

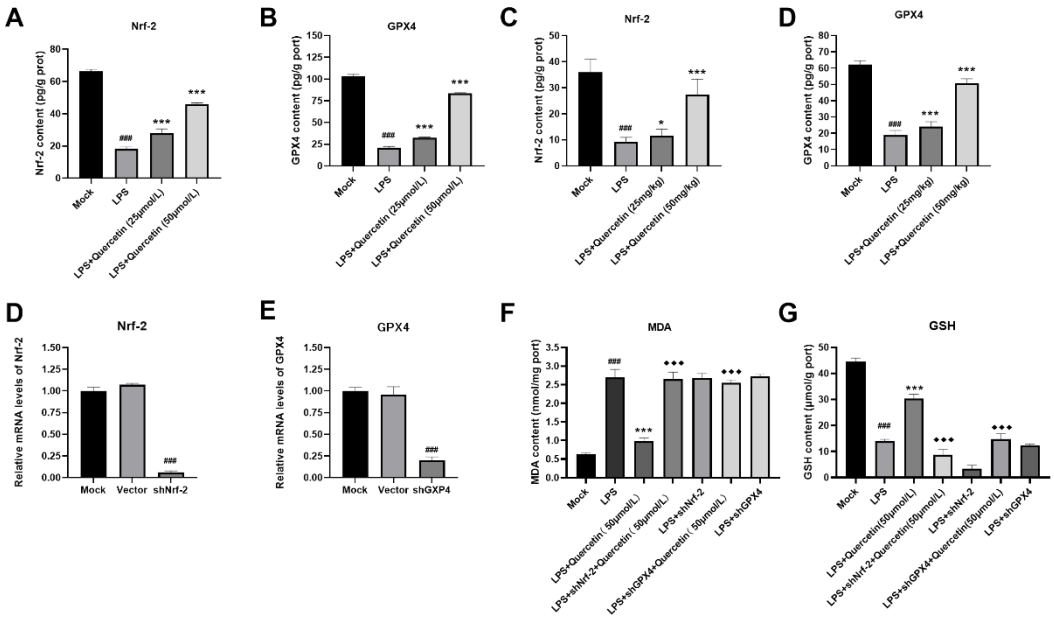

Figure 4: Quercetin Inhibits Ferroptosis Through the Nrf-2/GPX4 Signaling Pathway. (A-B) ELISA measurement of Nrf-2 (A) and GPX4 (B) protein levels in HK-2 cell homogenates. (C-D) ELISA measurement of Nrf-2 (C) and GPX4 (D) protein levels in homogenates from sepsis model mouse kidney tissues. (E-F) RT-PCR verification of the silencing effects of shRNA on Nrf-2 (E) and GPX4 (F). (G) Spectrophotometric measurement of MDA content in HK-2 cells. (H) Measurement of reduced GSH level in HK-2 cells by glutathione assay kit. LPS, lipopolysaccharide; MDA, malondialdehyde; GSH, Glutathione; ELISA, Enzyme Linked Immunosorbent Assay. In comparison to the Mock group, ### $p < 0.001$; relative to the LPS group, ***$p < 0.001$; relative to the LPS + Quercetin group, ◆◆◆$p < 0.001$.

GPX4 or using GPX4 inhibitors can increase lipid peroxidation and the deposition of ROS, thereby inducing ferroptosis.[26,27] Thus, the Nrf2/GPX4 pathway regulates cellular iron metabolism and ferroptosis. This study depicted that the level of Nrf2 and GPX4 was limited in cells undergoing iron death mediated by LPS, but this was reversed after intervention with quercetin. Moreover, the levels of MDA and GSH in HK-2 cells stimulated by LPS were not significantly affected by blocking the expression of Nrf2/GPX4 in these cells, indicating no synergistic effect, which suggests that the inflammatory response mediated by LPS or LPS itself may inhibit the Nrf2/GPX4 pathway. The Nrf2/GPX4 pathway's limited regulatory function is a crucial factor in S-AKI. Moreover, silencing the Nrf2/GPX4 genes could counteract the regulatory effects of quercetin on MDA and GSH content in HK-2 cells, depicting that the Nrf2/GPX4 pathway contributes to the process of quercetin treatment for sepsis-induced AKI. However, the mechanism through which quercetin modulates the Nrf2/GPX4 pathway is not yet precise and requires further study.

Despite the promising findings, several limitations of this study must be acknowledged. First, although the experimental results were significant, the results may not be as effectively applicable due to the small or insufficient sample size. To validate the current findings, large sample sizes will be required in future research. Second, while animal models and cell lines provided valuable insights into the protective effects of quercetin and Ferrostatin-1, the translational relevance to human clinical settings remains uncertain. Additional clinical trials need to confirm the effectiveness and safety of these treatments in human populations.

CONCLUSION

In summary, our research demonstrates that quercetin can antagonize septic renal injury by inhibiting renal tubular cell ferroptosis. Preliminary findings suggest that its mechanism is associated with the stimulation of the Nrf2/GPX4 pathway, providing some theoretical support for using quercetin to treat septic kidney injury.

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ABBREVIATIONS

S-AKI: Sepsis-associated Acute Kidney Injury; **LPS:** Lipopolysaccharide; **Cr:** Creatinine; **BUN:** Blood Urea Nitrogen; **IL:** Interleukin; **TNF-α:** Tumor Necrosis Factor-alpha; **MDA:** Malondialdehyde; **GSH:** Glutathione; **GPX4:** Glutathione Peroxidase 4; **Nrf-2:** Nuclear Factor Erythroid 2-Related Factor 2; **COX-2:** Cyclooxygenase-2; **ACSL4:** Acyl-CoA Synthetase Long-Chain Family Member 4; **FPN-1:** Ferroportin-1.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Therapeutic Effect of Quercetin on Sepsis-Induced Acute Kidney Injury

This study investigates the protective role of quercetin in alleviating sepsis-associated acute kidney injury (S-AKI) by suppressing ferroptosis. Quercetin demonstrated a significant improvement in renal function, as indicated by reduced levels of creatinine (Cr) and blood urea nitrogen (BUN), and lowered inflammatory markers (IL-1β, TNF-α, IL-6).

Mechanism of Action – Inhibition of Ferroptosis

Quercetin inhibits ferroptosis in renal tubular epithelial cells via the Nrf-2/GPX4 pathway. The study showed that quercetin upregulated ferroptosis-inhibiting genes (GPX4, FPN-1) and downregulated ferroptosis-promoting genes (ACSL4, COX-2), ultimately decreasing lipid peroxidation (MDA) and restoring antioxidant levels (GSH) in both animal and cell models.

Dose-Dependent Effects and Cellular Pathways

The effects of quercetin were dose-dependent, with both low (25 mg/kg) and high (50 mg/kg) doses significantly improving renal tissue morphology and function. The Nrf-2/GPX4 signaling pathway played a crucial role in mediating these protective effects. Silencing Nrf-2 or GPX4 genes diminished the protective effects of quercetin, further confirming the involvement of this pathway in quercetin's action against ferroptosis in S-AKI.

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