**INTRODUCTION**

Acute kidney injury (AKI), a serious kidney disorder with high morbidity and mortality, is characterized by a sudden decline in the glomerular filtration rate. AKI generally arises via three etiologies: prerenal, intrarenal, and postrenal causes. As a prerenal cause of AKI, renal ischemia/reperfusion injury (IRI) is a frequent and multifactorial complication after different diseases or medical events. Since the molecular mechanisms of AKI have been contemplated, inflammation has been recognized as a pivotal participant in IRI, and studies have focused on anti-inflammatory approaches. Interleukin-12 (IL-12) has been recognized as a proinflammatory factor involved during renal IRI. IL-12 is predominantly synthesized in macrophages and dendritic cells. In addition, renal tubular epithelial cells are an indispensable source of IL-12 in kidney disorders. IL-12 has a characteristic heterodimeric structure consisting of a heavy chain (p40) and a light chain (p35). The IL-12 p40 subunit is indispensable in the biological functions of IL-12, especially in immune reactions.

*Chrysanthemum indicum* L. (C. indicum, Compositae family) is widely distributed in East Asia and used in Chinese medicine. Linarin (LIN), an active ingredient in an herbal drug, can be extracted from *C. indicum*. The extensive medicinal value of LIN for its anti-reactive oxygen species (ROS) has also been reported to have anti-IRI effects on cardiac cells. This study aimed to demonstrate that LIN can protect the kidney from IRI.

**MATERIALS AND METHODS**

**Animal Protocol** LIN (CAS: 480-36-4) (purity ≥98.5%) was purchased from Extrasynthese (Lyon) and reconstituted into a sterile solution with dimethyl sulfoxide (DMSO). All procedures performed in studies involving animals were in accordance with the ethical standards of the Chinese Association for Laboratory Animal Care with the approval of the Animal Ethics Committee of the Affiliated Hospital of Qingdao University (AHQU-MAL2016016). Wistar rats (male, 6–8 weeks) were purchased from Vital River and randomly assigned to different groups, including a renal IRI group, a sham operation group, an ischemia/reperfusion injury-linarin (IRI-LIN) group and an ischemia/reperfusion injury-dissolvent (IRI-D) group. All rats were housed at a constant temperature of 23–24°C with 50% humidity and a light–dark cycle of 12:12 h. The rats in the IRI-LIN group received 60 mg/kg/d LIN orally for 7 d before IRI modeling, while the IRI-D group received dissolvent in the same volume. During the IRI modeling, bilateral renal vascular pedicles of the rats were clamped for 60 min using microvascular clamps (Roboz) and bloodstream restored unobstructed after ischemia. The rats in the sham operation group were treated in the same operation without clamping. After reperfusion for 24 h, blood samples were collected from
the venae cavae inferior. Meanwhile, samples of kidney tissue were collected. The concentrations of serum creatinine (SCr) and blood urea nitrogen (BUN) were determined using Creatinine Assay Kit (Nanjing Jiancheng Bioengineering Institute).

**Histopathology Analysis** Kidney tissue samples were collected after IRI and fixed in 10% formaldehyde at 4°C overnight. The samples were embedded in paraffin and cut into 5-µm-thick sections. Then, sections were stained with hematoxylin (Solarbio) and eosin (Solarbio) (H&E). The H&E staining images were observed and captured using an optical microscope (DP73; Olympus, Tokyo, Japan).

**Cell Culture and Hypoxia Reoxygenation (H/R) Treatment** HK2 human renal tubular epithelial cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were derived from a population from the American Type Culture Collection and were authenticated by short tandem repeat analysis. All cells were recovered and passaged for six generations before all experiments. The HK2 cells were plated in 6-well cell culture plates (1×10^6 cells/well) in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin liquid (100 U/mL penicillin, 0.1 mg/mL streptomycin) in a humidified atmosphere containing 5% CO₂ at 37°C and grown to 70% confluence. The cells were then cultured in glucose-free DMEM without FBS in a hypoxic atmosphere containing 1% O₂, 5% CO₂ and 94% N₂, followed by reoxygenation in complete medium in normoxia (5% CO₂). The entire H/R process was conducted at 37°C. Cells in the control group were not subjected to H/R. LIN solutions at different concentrations were prepared for co-incubation with HK2 cells, and equal volumes of DMSO were added to the solvent control groups during H/R.

**Cell Viability Detection** HK2 cells in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin-streptomycin liquid were distributed in 96-well cell culture plates (5×10^3 cells/well). After overnight cultivation, cells in different wells were cultured with graded concentrations of LIN or DMSO for 48 h. Then, the medium containing the drugs was completely replaced with 100 µL of DMEM/F12 medium, and 10 µL of Cell Counting Kit-8 (CCK8) solution (MedChemExpress) was added to each well. The optical density at 480 nm was read with a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) after a 2 h culture. The cell viability was determined by the absorbance ratio between LIN-exposed and DMSO-exposed cells.

**Quantitative Real-Time RT-PCR (qRT-PCR)** Total RNA was extracted from 10mg tissue samples using 1mL TRIzol Reagent (Invitrogen, Waltham, MA, U.S.A.) or HK2 cells in 6-well plates using 500 µL/well TRIzol Reagent (Invitrogen). Total RNA was subjected to cDNA synthesis catalyzed by PrimeScript RT Enzyme Mix (TaKaRa Bio, Shiga, Japan). Then, qRT-PCR was performed in a CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, U.S.A.) using a SYBR ExScript qRT-PCR Kit (TaKaRa Bio). The primers used are shown in Table 1. qRT-PCR was repeated 3 times, and the expression levels of target genes were analyzed using the comparative threshold cycle method (2−ΔΔCT).

**Identification of Differentially Expressed Genes (DEGs) According to Microarray Analysis** Raw microarray data were downloaded from GEO DataSets (https://www.ncbi.nlm.nih.gov/gds). Kidney samples from mice with renal IRI were collected 4h after reperfusion, and kidney samples from corresponding sham mice were selected from GSE98622 (Illumina HiSeq 2000 Mus musculus platform). The microarray data were contributed by Liu et al. and downloaded for further analysis. The R (version 3.3.4) programming language was used for microarray data analysis, which included quality assessment and normalization of the data and identification of DEGs. The raw data were preprocessed using a robust multi-array averaging algorithm. (14) The Linear Models for Microarray Analysis package was loaded for differential expression analysis. The adjusted p value (adj.p) was calculated with the Benjamini & Hochberg method. For final identification of DEGs, absolute log₂FC > 2 with adj.p < 0.05 was considered the threshold.

**Protein–Protein Interaction (PPI) Network Analysis** PPI information was acquired from the STRING database (http://www.string-db.org/). Medium confidence (0.4) was chosen as the minimum required interaction score to screen the interactions among DEGs. PPI network visualization was conducted by Cytoscape (version 3.7.2) software. Hub genes were selected by density maximum neighborhood component methods. (15)

**Transfection of Small Interfering RNA (siRNA)** HK2 cells were plated into 6-well culture plates in DMEM/F12 medium containing 10% FBS and 1% penicillin-streptomycin liquid. When grown to 60% confluence, the cells were transfected with siRNA against target gene or control siRNA (Dharmacon, Thermoscientific) using Lipofectamine RNAiMAX (Life Technologies, DriveRockville, MD, U.S.A.) according to the manufacturer’s instructions. After 48h, knockdown of target gene expression was validated by qRT-PCR analysis and cultures with more than 80% reduction in target gene mRNA expression comparing to the control cultures were included in further experiments.

**Molecular Docking** Molecular docking was carried out by Auto Dock 4.2.6 (The Scripps Research Institute) and followed the description of Hong et al. (16) Briefly, proteins encoded by the hub genes were selected as rigid receptor molecules, while LIN was selected as the ligand for protein docking. PDB

| Gene      | Forward primer             | Reverse primer             |
|-----------|-----------------------------|-----------------------------|
| ETS2      | TGGAGACCGATGGGAGTTTAA       | CCCGAGCTTTGTTGATGAT         |
| IL-12 p35 | GATGGCCCTCTTGCCTTAGTAGT     | GCCGTGACATGCTCATCAATAA      |
| IL-12 p40 (human) | GATGGTGCCAGGGAGGACGT | AGTCTTGGGTGGTGGTCAGTTT     |
| IL-12 p40 (rat) | GGTGCCAGAAAACACGGACCTT | TGGCAGCCACTTGAATCTC        |
| KIM-1     | ACTCTCTGAAGCTGGAAATGG       | CAAAGCTCAGAGGCCTCCATC      |
| β-Actin   | CGTTGACATCCGTAAAGACCTC     | TAGGAGCCAGGGGCAGTAATCT     |

Table 1. Primers Used in qRT-PCR
files of proteins were downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). In addition, the SDF file for LIN was downloaded from the NCBI PubChem Compound database (https://www.ncbi.nlm.nih.gov/pccompound) and translated into PDB file format by Open Babel GUI\(^1\); both of the structure files were improved by adding compensatory atoms. Then, a pdbqt file for the ligand was established with parameters including atomic coordinate, atom type, electric charge and torsion bond information. After the receptor molecule and ligand files were prepared, a grid box was set for the movement and rotation of the ligand. Finally, docking was accomplished via the AutoDock4 program.

**Statistical Analysis** Data from experiments were analyzed by a t-test for comparisons between two groups or by ANOVA for comparisons among multiple groups. Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.) was used to perform all statistical calculations (\(p < 0.05\) was considered statistically significant) and create the histograms and line charts.

**RESULTS**

**LIN Protects Kidney from IRI** Kidney injury molecule 1 (KIM-1), a biomarker of kidney injury was applied to evaluate the effect of LIN on IRI kidney samples. As shown in Figs. 1A–D, the renal tissue as well as the renal function were obviously spoiled after IRI according to H&E staining, KIM-1 mRNA expression level, SCr level, BUN level and IL-12 p40 mRNA level in kidney samples of different groups. The experiments were repeated three times. *\(p < 0.05\) compared with the sham group. # \(p < 0.05\) compared with the IRI group. (Color figure can be accessed in the online version.)

**Toxicity of LIN to Normal HK2 Cells** The toxicity of LIN on HK2 cells was evaluated before the functional experiments in vitro. There was no significant difference in the cell activity between the LIN and DMSO groups after incubation with LIN (≤ 50 \(\mu\)M) for 48 h (Fig. 2A). These results indicated that the toxicity of LIN to normal HK2 cells was negligible at concentrations of 50 \(\mu\)M or less.

**LIN Decreased the mRNA Expression Level of IL-12 p40** The H/R model was successfully established with
human renal tubular epithelial cells (hypoxia for 24 h and reoxygenation for 1 h). In the H/R model, the IL-12 p40 expression level showed little but not significantly reduce under incubation with 20 μM LIN during H/R, in contrast to that in cells cultured without LIN; however, significant decrease in IL-12 p40 was observed under incubation with 30 μM and 40 μM LIN. In contrast, the expression level of IL-12 p35 did not change significantly under incubation with either 20 μM, 30 μM or 40 μM LIN (Fig. 2B).

The E26 Oncogene Homolog 2 (ETS2) Was Selected as a Hub Gene According to Differential Expression Analysis and PPI. Via bioinformatics, we identified 186 DEGs (Table S1) after analysis of the raw data from IRI and normal kidney samples. Eleven hub genes were selected from the DEGs, and interactions of the eleven hub genes are shown in Fig. 3. Among the hub genes, ETS2 is indicated by the arrow in purple. (B) ETS2 expression was continuously upregulated in human renal tubular epithelial cells during IRI. The experiments were repeated three times. *p < 0.05 compared with cells cultured under normal conditions. (Color figure can be accessed in the online version.)

The Effect of LIN on IL-12 p40 Expression during H/R Was Inhibited by the Knockdown of ETS2 Expression. The knockdown of ETS2 expression was successfully conducted (Fig. 5A). Comparing with the group only exposed in H/R, the expression of IL-12 p40 induced by H/R was significantly reduced in the group transfected siRNA against ETS2. While the IL-12 p40 expression showed no significant difference between the ETS2 knock down group and 40 μM LIN treated ETS2 knock down group after hypoxia for 24 h and reoxygenation for 1 h (Fig. 5B).

The Binding of LIN and ETS2 Was Predicted by Molecular Docking. Molecular docking was employed to explore the probability of binding between LIN and ETS2. The simulated binding structures of LIN (pink) and ETS2 (yellow) are shown in Fig. 6. As a ligand, LIN could bind to the surface of ETS2 with a binding energy of −5.8 (Fig. 6A). To
show the details of binding, the structure was also simulated in a cartoon (Fig. 6B). The hydrogen bonds are displayed as marine blue dashed lines. Arginine, glycine, isoleucine and glutamine, which were recognized as polar residues in contact with LIN were located on a conserved area according to amino acid sequence alignment of ETS2 (Fig. 7A). According to the SMART database (https://smart.embl.de/), the binding area is a part of the SAM PNT domain (Fig. 7B).

**DISCUSSION**

Herbal drugs have been extensively produced and widely used in East Asia. To augment therapy for IRI, an increasing variety of herbal drugs has been manufactured. As an extract of *C. indicum*, LIN has been widely studied in different medicinal fields.19,20 The effective use of LIN for anti-IRI therapy was demonstrated based on research on cardiac disease.13 In the current study based on the IR model, we found that LIN
could also protect kidney from IRI and inhibit the upregulation of IL-12 p40 induced by IR. In renal IRI, renal tubular epithelial cells are not only victims but also triggers especially of inflammation. To explore the protective mechanism of LIN, an H/R model of HK2 cells was established, and the upregulation of IL-12 p40 expression induced by H/R was inhibited by “larger” doses of LIN (30 and 40 μM), the result was consistent with the in vivo experiment.

As a classic proinflammatory factor, IL-12 can regulate the development, proliferation and differentiation of helper T 1 cells and can also induce the killing activity of natural killer cells through the enhancement of interferon-γ production. In the study conducted by de Paiva et al., bioactive IL-12 and its target cells, helper T 1 cells, are involved in inflammation during renal IRI via the knockout of IL-12. Therefore, the reduction in IL-12 bioactivity is very important for the inhibition of renal IRI, and the depression of IL-12 bioactivity may be the possible protective mechanism of LIN in renal IRI.

Recognition of DEGs between normal and IRI kidney samples is an efficient approach for identifying the key regulators of the complex process. To explore the possible equestroN of IL-12, we downloaded and analyzed raw microarray data. After PPI analysis, ETS2, an upregulated gene after renal IRI, was noted. ETS2 is a transcription factor that can be detected in different organs, including the spleen, small intestine and kidney. The functional diversity of this protein has been shown in different research fields. In many studies, ETS2 has been characterized as a regulator in different kinds of tumors. Inhibition of ETS2 can prevent the invasion and metastasis of renal cell carcinoma cells though the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. In addition, ETS2 is involved in kidney development. Indeed, binding sites for ETS2 were previously found in the promoter of human IL-12 p40, and the actual activity of ETS2 toward IL-12 p40 was confirmed by Sun et al. In our study, we demonstrated the critical role of ETS2 in IL-12 p40 inhibition of LIN during H/R via knockdown of ETS2 and predicted a possible interaction between LIN and ETS2 using molecular docking. The contact area of ETS2 is located on the SAM PNT domain, the SAM PNT domain is a highly conserved domain that is important for the PPI between ETS2 and other proteins. Studies had proved that ETS2 acts synergistically with other proteins in the regulation of IL-12 p40 such as histone acetyltransferase p300 and nuclear factor-kappaB (NF-κB). Our study indicated that LIN may bind to the SAM PNT domain and change the conformation of the domain, which could inhibit the interaction between ETS2 and synergistic proteins and finally depress the auxo-action of IL-12 p40. Although specific mechanisms and LIN binding site still need further experiments, we confirmed that LIN could protect kidney against IRI via the inhibition of ETS2/IL-12.

In summary, in our study, downregulation of IL-12 p40 expression during IRI indicated that LIN could be a candidate anti-inflammatory drug to protect renal tubular epithelial cells against IRI. Although LIN has shown potentially differential anti-IRI effects, more clinical evidence is necessary before LIN can be used in renal IRI therapy.

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Conflict of Interest The authors declare no conflict of interest.
Supplementary Materials  The online version of this article contains supplementary materials.

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