Frontiers in Bioscience Landmark

*Front. Biosci. (Landmark Ed)* **2026**; 31(8): 52184
https://doi.org/10.31083/FBL52184

*Original Research*

# Itaconic Acid and Its Isomers Ameliorate Renal Ischemia-Reperfusion Injury in Rats by Inhibiting NLRP3-Mediated Inflammation

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Academic Editors: Moo-Ho Won and Graham Pawelec
Submitted: 23 March 2026 Revised: 23 June 2026 Accepted: 8 July 2026 Published: 17 August 2026

**Abstract**

**Background:** Itaconic acid and its isomers citraconic acid and mesaconic acid are a recently identified class of metabolites with anti-inflammatory and antioxidant effects. This study investigates their roles in ischemia-reperfusion-induced acute kidney injury. **Methods:** In this study, a rat model of renal ischemia-reperfusion injury (IRI) was established, and itaconic acid, citraconic acid and mesaconic acid were administered as a preoperative intervention. After the operation, renal injury was evaluated by contrast-enhanced ultrasound, biochemical analyses and histopathological staining. **Results:** The intervention with itaconic acid and its isomers reduced the production of the inflammatory cytokines interleukin-18 (IL-18) ($p < 0.05$), inhibited activation of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome ($p < 0.05$), and reduced reactive oxygen species levels ($p < 0.05$). The intervention with itaconic acid and mesaconic acid reduced the production of the inflammatory cytokine interleukin-1β (IL-1β) ($p < 0.05$). The expression of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) spot protein in the intervention group was significantly lower than that in the renal ischemia-reperfusion injury (IRI) group ($p < 0.01$). **Conclusions:** These findings suggest that itaconic acid, citraconic acid, and mesaconic acid may be potential therapeutic agents for renal ischemia-reperfusion through their anti-inflammatory and antioxidant effects.

**Keywords:** itaconic acid; citraconic acid; mesaconic acid; renal ischemia-reperfusion injury; contrast-enhanced ultrasound

## 1. Introduction

Acute kidney injury (AKI) is a common disease (8–16% of hospital admissions) and a serious disease (a four-fold increase in hospital mortality), whose global incidence is growing. The high cost of treatment in the later stages of the disease and the risk of chronic kidney disease have made it a growing global health problem [1]. The main pathologic feature of AKI is a decreased glomerular filtration rate, resulting in a short period of reduced kidney function, which is characterized by tubular and vascular damage, as well as uncontrolled inflammatory responses [2]. However, the current therapeutic strategies for AKI remain unsatisfactory in clinical practice. AKI is related to surgery, drug therapy and decreased renal perfusion pressure, in which renal ischemia-reperfusion injury (IRI) is a major cause of acute renal injury and secondary renal function loss [3]. Given the rapid progression of AKI, early diagnosis is crucial to reduce AKI-associated mortality. Current diagnosis of acute kidney injury relies on measurements of serum creatinine (Cr) levels and urine output. However, the serum Cr level is affected by many factors such as diet and drug therapy, and the specificity and sensitivity of measuring urinary volume are not sufficient to be a diagnostic marker of AKI. These limitations may lead to an underestimation of the extent of damage to renal function. Given the serious consequences of AKI, an increasing number of researchers are focusing on early identification of patients with AKI, aiming to improve their outcomes [4,5]. At present, ultrasound technology has been increasingly applied to the diagnosis of various diseases [6], and it is a common means for clinical application to screen or diagnose diseases, especially in urology and other systems.

Contrast-enhanced ultrasound (CEUS) is a quantitative imaging technique. Following the administration of contrast agents, high-echo microbubbles are used to enhance the contrast between the target region and surrounding tissue, which can more accurately reflect blood perfusion and increase the sensitivity and specificity of blood flow measurement [7,8]. Currently, the main component of clinically used ultrasound contrast agents is sulfur hexafluoride (SF6) microbubbles, which are not affected by glomerular filtration rate and tubular reabsorption, have no metabolic burden on the kidney, and do not increase the risk of kidney injury [9,10]. Therefore, compared with enhanced computed tomography or enhanced magnetic resonance imaging, CEUS has significant advantages in the diagnosis of kidney disease and can safely assess renal perfusion and microcirculation in critically ill patients [11,12]. In addition, the time-intensity curve (TIC) and quantitative parameters (i.e., peak intensity [Peak], time to peak [Tp], area

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under the curve [AUC] and mean transit time [MTT]) were obtained by quantifying the change in time-concentration of contrast agents in the region of interest (ROI). These are sensitive indicators of renal blood perfusion, which may help to assess renal tissue damage in the early stages of AKI [13,14,15]. It has been proven that CEUS examination combined with TIC curve analysis has high application value in evaluating renal blood perfusion in IRI [16,17].

The pathological mechanism of renal IRI involves multiple factors, such as oxidative stress, inflammation, apoptosis, and autophagy, which lead to vascular dysfunction, immune system activation, and renal tubular epithelial cell injury [18]. During ischemia, mitochondrial synthesis of Adenosine 5′-triphosphate (ATP) is impaired, leading to cell death; after ischemic tissue reperfusion, mitochondria generate excessive amounts of reactive oxygen species (ROS), which directly oxidize cells and stimulate the release of pro-inflammatory mediators, leading to tissue injury [19]. Therefore, reducing ROS accumulation can effectively alleviate renal IRI [20,21,22]. Due to the multifactorial pathophysiological mechanisms of renal IRI, there is no effective pharmacological intervention to prevent or reverse the condition [1]. Current studies have proposed dialysis treatment [23], alternative therapy [24], stem cell therapy [25,26,27], etc., but these treatments all have certain limitations. Based on this, a growing body of research is focusing on pharmacological interventions for AKI [28,29,30].

Itaconic acid is a recently discovered endogenous metabolite, which is mainly involved in the regulation of cellular metabolism, and plays an important role in cellular immunity and inflammatory metabolism [31,32,33,34,35]. It has antibacterial, anti-inflammatory and antioxidant properties [36,37,38,39]. First, itaconic acid can reduce ROS production by inhibiting the mitochondrial tricarboxylic acid (TCA) cycle and succinate dehydrogenase (SDH) activity [40]. Second, itaconic acid is also released into the cytoplasm, where it can modify Kelch-like ECH-associated protein 1 (Keap1), activate nuclear factor erythroid 2-related factor 2 (Nrf2), and promote the expression of anti-inflammatory and antioxidant genes [41]. In addition, itaconic acid can also induce the release of cytochrome c by inhibiting the activity of cytochrome c oxidase (Complex IV) in the respiratory chain, which stimulates the opening of the mitochondrial permeability transition pore, thereby reducing the accumulation of intracellular $Ca^{2+}$ [42]. Itaconic acid's inhibition of NLRP3inflammasome is also considered to be an important part of its anti-inflammatory mechanism [32]. It has been shown that the itaconic acid derivative 4-octyl itaconate (4-OI) can ameliorate early renal fibrosis by inhibiting inflammation and reducing oxidative stress [43,44].

Although citraconic acid and mesaconic acid, as isomers of itaconic acid, have similar biological properties [45], there is still a gap in their preclinical studies and the mechanism of action of itaconic acid in the acute phase of renal injury remains elusive. To this end, the present study established a renal IRI-induced AKI model to systematically investigate the therapeutic effect of exogenous itaconic acid on AKI and the efficacy of its two isomers in renal IRI. Our results preliminarily suggest that both itaconic acid and its analogs exhibit anti-inflammatory effects after renal ischemia/reperfusion and enhance the antioxidant capacity of renal cells. Furthermore, our findings indicate that ultrasonography is an effective tool for assessing renal IRI and the efficacy of pharmacological interventions.

## 2. Materials and Methods

### *2.1 Experimental Method*

#### 2.1.1 Main Reagents and Instruments

Itaconic acid (C11530755, Macklin, Shanghai, China); Citraconic acid (C39535F63, ACMEC, Shanghai, China); Mesaconic acid (C15385206, Macklin, Shanghai, China); Xylazine (Selma Biological Company, Shenzhen, China); Tiletamine Hydrochloride and Zolazepam Hydrochloride for Injection, Zoletil® 50 (Virbac, France); Sodium Chloride Injection (H51022438, Meida Kangjiale Pharmaceutical Co., Ltd., Sichuan, China); Hematoxylin Staining Solution (Servicebio, Wuhan, China); Eosin Staining Solution (Bomei Biotechnology, Hefei, China); DAPI Staining Reagent (G1012, Servicebio, Wuhan, China); Phosphate-buffered saline (PBS) Buffer (ZLI-9062, Zhongshan Goldenbridge Biotechnology, Beijing, China); Primary Antibody: Anti-ASC (A1170, Aibotek, Wuhan, China); Secondary Antibody: FITC-conjugated Goat Anti-Rabbit IgG (Cat# GB22303, Servicebio, Wuhan, China); Probe: Dihydroethidium (DHE) (Cat# S0063, Beyotime Biotechnology, Shanghai, China); TUNEL Kit (1168479590, Roche Group, Switzerland); ELISA Kits: Rat IL-1β ELISA Kit (ZC-36391), Rat IL-18 ELISA Kit (ZC-36389), Rat KEAP1 ELISA Kit (ZC-56646), Rat Nrf2 ELISA Kit (ZC-36695), Rat SDH ELISA Kit (ZC-36707), Rat NLRP3 ELISA Kit (ZC-54271) (Zocai Biology, Shanghai, China).Siemens Ultrasound Diagnostic System (ACUSON S3000, Siemens Medical Solutions USA, Inc, Mountain View, CA, USA); D3024R Centrifuge (DLAB Scientific Inc., China); BS-2200M Biochemistry Analyzer (Mindray Medical, China); Leica RM2016 Rotary Microtome (Leica Microsystems, Germany); JT-12S Automatic Tissue Dehydrator (Junjie Electronics, Wuhan, China); BMJ-A Tissue Embedding Center (Zhongwei Electronic Instrument Factory, Changzhou, China); PHY-III Pathological Tissue Flotation and Drying Oven (Zhongwei Electronic Instrument Co., Ltd., Changzhou, China); Pannoramic 250 Digital Slide Scanner (3DHISTECH, Hungary); VS200 Digital Slide Scanner (OLYMPUS, Japan); SpectraMAX Plus384 Microplate Reader (Molecular Devices, San Jose, CA, USA); KZ-III-F High-Speed Low-Temperature Tissue Grinder (Servicebio, Wuhan, China).

2.1.2 Experimental Animals and Grouping

The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University. A total of 61 healthy adult male Sprague-Dawley rats (weighing 250 ± 50 g; aged 8~10 weeks) were provided by the Animal Experimental Center of Southwest Medical University (license number: SCXK(Sichuan)2023-0017). The rats were randomly divided into five groups: (1) The Sham group (n = 10) received 2 mL/kg/d of normal saline via injection for three days before surgery. (2) The Renal Ischemia-Reperfusion (IRI) group (n = 12) received intraperitoneal injections of 2 mL/kg/d of normal saline for 3 days before surgery. (3) Renal IRI+ itaconic acid intervention group (Itaconic acid group), n = 13, each rat was given itaconic acid (C11530755, Macklin, Shanghai, China) 50 mg/kg/day [43] by intraperitoneal injection for 3 days before surgery. (4) Renal IRI+ citraconic acid intervention group (Citraconic acid group), n = 13, each rat was given 50 mg/kg/ day by intraperitoneal injection (C39535F63, ACMEC, Shanghai, China) for 3 days before surgery. (5) The Renal IRI + Mesaconic Acid group (n = 13) was administered 50 mg/kg/d of mesaconic acid via intraperitoneal injection (Cat# C15385206, Macklin, Shanghai, China).

2.1.3 Establishment of an Animal Model

Before surgery, the experimental rats were fasted for 12 hours but had free access to water. They were anesthetized via intraperitoneal injection of Xylazine (11.25 mg/kg, 5 mg/mL) and Zoletil® 50 (45 mg/kg, 5 mg/mL). The skin and muscle layers were cut along the right waist and the right kidney was ligated and excised. The left kidney pedicle was freed and the kidney pedicle was clipped with a non-invasive artery clamp. Following the observation of renal color change, the kidney was returned to the abdominal cavity, and the incision site was maintained at body temperature and kept moist for 45 minutes to establish the ischemia model. After 45 minutes, the arterial clip was released, and the color change of the kidney was observed. After confirming that the kidney was reperfused, the incision was closed layer by layer. After the operation, the rats were kept clean and fed freely. The left kidney of the rats in the sham group was only freed of the kidney pedicle, and no arterial clamp was used to clamp the kidney pedicle, and other operations were the same as those in other groups.

At the designated experimental endpoint, all rats were euthanized to minimize suffering. The procedure was performed under deep anesthesia, which was induced and maintained with Xylazine (11.25 mg/kg, 5 mg/mL) and Zoletil® 50 (45 mg/kg, 5 mg/mL). The depth of anesthesia was carefully monitored by assessing the absence of the pedal withdrawal reflex and the palpebral reflex. Once a surgical plane of anesthesia was confirmed, the animals were euthanized by cervical dislocation. This protocol was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of the Affiliated Hospital of Southwest Medical University.

2.1.4 Contrast-Enhanced Ultrasound (CEUS)

All the rats were examined by ultrasound 24 hours after the operation, and the channels for injecting ultrasound contrast agent and normal saline were established in advance. The Sonovue contrast agent was reconstituted as an SF6 microbubble suspension with 5 mL of normal saline and agitated repeatedly before use. A Siemens ACUSON S3000 ultrasonic diagnostic instrument (Siemens GmbH) and a 9L4 variable-frequency linear array probe were used, with parameters set as follows: frequency 4.0 MHz, depth 3.5 cm, and mechanical index 0.07. The contrast agent was injected rapidly at a dose of 0.4 mL/kg through the tail vein catheter, followed by a 0.2 mL normal saline flush. The left kidney was observed continuously. The renal cortical region with an area of 0.05 $mm^2$ was delineated as the ROI, and the analysis software auto-tracking contrast quantification (ACQ) was used for quantitative analysis. The relevant parameters (Peak, Tp, AUC, MTT) were calculated, and the corresponding TIC curves were generated. All parameters were measured three times by the same operator.

2.1.5 Serum Biochemical Analysis

After CEUS, 3 mL of blood was collected from the heart and the serum was harvested from the supernatant after centrifugation. Serum Cr, Urea, and Cystatin C (Cys-C) levels were measured using a Mindray BS-2200M automatic biochemical analyzer.

2.1.6 Hematoxylin-Eosin (HE) Staining

After blood collection, the rats were euthanized, and the left kidney tissues were quickly excised, half of which were fixed in fixative solution and stored at 4 °C, and half of which were frozen in liquid nitrogen and stored in a freezer at –80 °C for subsequent analysis. The specimens underwent paraffin embedding, and HE staining was performed after sectioning. Pathological morphological changes of rat kidney sections were observed under the microscope.

2.1.7 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of Interleukin (IL)-1β, IL-18, NLRP3, Keap1, Nrf2, and SDH were quantified by ELISA. Rat kidney tissues were accurately weighed after the removal of residual blood. PBS was added at a mass-to-volume ratio of 1:9, followed by homogenization using a tissue grinder. The homogenate was centrifuged at 5000 ×g for 10 min, and the supernatant was collected for analysis. The actual concentration of the sample was measured and calculated according to the kit instructions.

2.1.8 Dihydroethidium (DHE) Staining

The frozen sections of the rat kidney were restored to room temperature and then labeled and stained accord-

ing to the instructions of the DHE detection kit (S0063, Beyotime Biotechnology, Shanghai). The frozen sections were restored to room temperature and washed on a rotary shaker three times in PBS (ZLI-9062, Beijing Zhongshan Jinqiao Biotechnology Co., LTD., 0.01 mol/L, pH 7.4) for 5 minutes each time. An appropriate volume of DHE solution was added to each tissue section, and the slides were protected from light. The sections were then incubated at 37 °C for 30 minutes. The incubated tissue sections were washed three times with PBS, each time for 5 minutes (in the dark). 4',6-diamidino-2-phenylindole (DAPI, G1012, Servicebio) was added dropwise, and the sections were incubated at room temperature for 10 minutes. The sections were washed with PBS three times, each time for 5 minutes. The slides were sealed with an anti-fade mounting medium. Scanning and browsing software was used for image acquisition of sections. Each section was initially examined at low magnification, followed by the acquisition of images at 100× and 200× magnification. A total of 3 fields of view were collected. The proportion of positive area per image was calculated using the Halo 101-WL-HALO-1 data analysis system (Indica Labs, Albuquerque, NM, USA).

#### 2.1.9 Immunofluorescence Staining of Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain (ASC)

ASC is an important component protein of the NLRP3 inflammasome. To further verify whether itaconic acid and its isomers, citraconic acid and mesaconic acid, affect inflammasome activation, immunofluorescence staining for ASC was performed on frozen sections of rat kidney according to the kit instructions. The primary antibody was anti-ASC (Aibotek, A1170; diluted 1:100), and the secondary antibody was FITC-conjugated goat anti-rabbit IgG (Servicebio, GB22303; diluted 1:100). Frozen sections were warmed up at room temperature and fixed, followed by immersion in citrate buffer (ZLI-9065, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; pH 6.0) for antigen retrieval, blockade of endogenous peroxidase, and blocking with bovine serum albumin (GC305010, Servicebio). The blocking solution was gently removed. The primary antibody, prepared in PBS at the appropriate dilution, was added to the sections. The sections were placed horizontally in a humidified chamber and incubated at 4 °C overnight. After washing three times with PBS, the secondary antibody was added dropwise, followed by another three washes with PBS. DAPI was then added dropwise and incubated at room temperature for 10 minutes. Finally, the sections were washed three times with PBS and mounted using an anti-fade mounting medium. Following staining, images of the sections were acquired using scanning and viewing software. Each section was observed at low magnification before examining all tissues, after which images were captured at 100× and 200× magnification. A total of three fields of view were collected per section. The Halo data analysis system was used to calculate the proportion of positive area in each image.

#### 2.1.10 TdT-Mediated dUTP Nick End Labeling (TUNEL)

The renal tissue of rats subjected to TUNEL staining was examined under a fluorescence microscope to assess the breakdown of nuclear DNA during apoptosis and determine the percentage of apoptotic cells. The fixed tissue was dehydrated by an automatic dehydrator, then the specimens were embedded and stained according to the TUNEL test kit (1168479590, Roche Group). The samples were washed with PBS three times for 5 minutes each. The nuclei were counterstained with DAPI for 15 minutes, rinsed with PBS, mounted with glycerin gelatin, and stored at –20 °C. Sections were scanned using a Pannoramic 250 digital slide scanner. Image collection and apoptotic cell analysis were carried out. Subsequently, the percentage of apoptotic cells was calculated via manual counting of the images.

### *2.2 Statistical Analysis*

Statistical analysis was performed using IBM SPSS Statistics 27.0 software (IBM Corp., Armonk, NY, USA), and the measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The Shapiro-Wilk test was used to assess the normality of the data distribution before the analysis. One-way Analysis of Variance (ANOVA) and the Kruskal-Wallis test (for non-parametric data) were used to compare indicators across the study groups. A $p$-value of less than 0.05 was considered statistically significant.

## 3. Results

### *3.1 CEUS Parameters Assessed Renal Function Changes*

We compared the IRI group with the Sham group to confirm the effect of renal IRI on renal function in rats, and compared the itaconic acid-, citraconic acid-, and mesaconic acid-treated groups with the IRI group to investigate whether these substances have protective effects on renal IRI. Fig. 1A shows the experimental design of itaconic acid and its isomers, citraconic acid and mesaconic acid for the intervention in IRI-induced AKI. Fig. 1B shows the changes in renal coloration during the establishment of the IRI model upon confirming renal ischemia and reperfusion.

First, the renal CEUS parameters were statistically analyzed in each group. The results showed that compared with the sham group, the peak value of the IRI group was increased ($p < 0.001$), while the peak values of the itaconic acid group, citraconic acid group, and mesaconic acid group were significantly decreased relative to the IRI group ($p < 0.001$) (Fig. 1C). Compared with the sham group, the Tp value of the IRI group was increased ($p < 0.01$). The Tp value of the mesaconic acid group was decreased compared with the IRI group ($p < 0.05$), but the difference in Tp value between the itaconic acid group and the citraconic acid group versus the IRI group was not statistically signif-

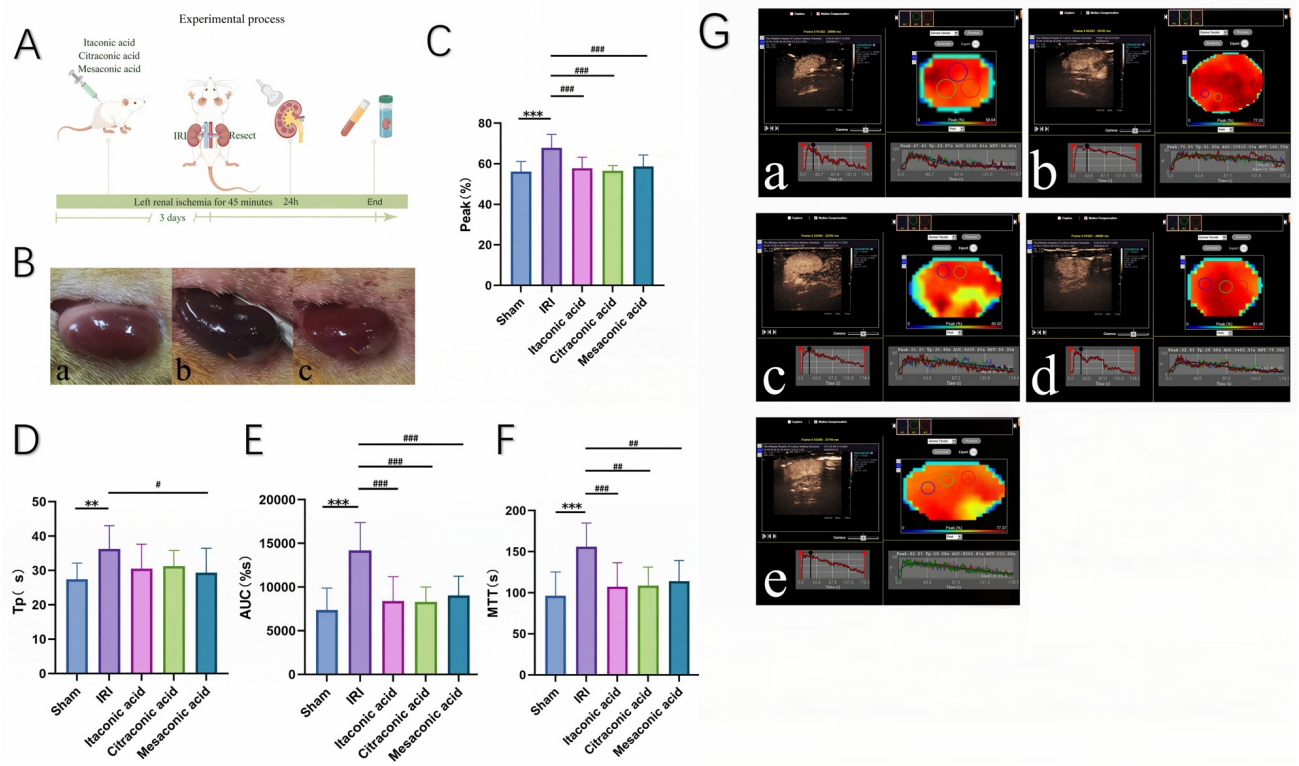



**Fig. 1. CEUS parameters assess renal function changes**. (A) Schematic diagram of experimental procedure. Healthy male SD rats were treated with itaconic acid, citraconic acid and mesaconic acid respectively, and a unilateral renal ischemia (45 min) and reperfusion (24 h) model was established on the third day. 24 h later, CEUS was examined, blood was drawn and kidney samples were taken (By Figdraw). (B) The renal IRI model in rats was established to induce renal color changes. (a) Before ischemia; (b) Ischemia; (c) Reperfusion. (C–F) Results of quantitative parameters of renal CEUS (Peak, Tp, AUC, MTT) in rats. One-way ANOVA was used for Peak value and AUC value data, and a non-parametric test was used for Tp value and MTT value data, with n = 10–13. Remarks: Compared with sham group: $^{**}p < 0.01$, $^{***}p < 0.001$; Compared with IRI group: $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$. (G) Renal cortex perfusion TIC curve of rats in each group. Tp reflects the time from the start of the injection of the contrast agent to the Peak intensity. Peak represents the signal intensity when the contrast agent in the ROI reaches its peak. MTT reflects the time from the start of the injection of the contrast agent to the clarification reaching half of the peak intensity. AUC is the cumulative total integral value of the contrast agent over a period of time within the reflected ROI. (a) Sham group; (b) IRI group; (c) Itaconic acid group; (d) Citraconic acid group; (e) Mesaconic acid group. CEUS, contrast-enhanced ultrasound; SD, Sprague-Dawley; Tp, time to peak; Peak, peak intensity; AUC, area under the curve; MTT, mean transit time; ANOVA, Analysis of Variance; IRI, ischemia-reperfusion injury; TIC, time-intensity curve; ROI, region of interest.

icant ($p > 0.05$) (Fig. 1D). Compared with the sham group, the AUC value in the IRI group was significantly increased ($p < 0.001$), while the AUC value in the itaconic acid group, citraconic acid group and mesaconic acid group was significantly decreased compared with the IRI group ($p < 0.001$) (Fig. 1E). Compared with the sham group, the MTT value in the IRI group was prolonged ($p < 0.001$), and MTT values in the itaconic acid group ($p < 0.001$), citraconic acid group ($p < 0.01$) and mesaconic acid group ($p < 0.01$) were shortened compared with the IRI group (Fig. 1F). The TIC curves showed that the signal strength of the contrast agent in the sham group and drug intervention group rapidly decreased to the baseline level after reaching the peak, while that of the contrast agent in the IRI group decreased slowly after reaching the peak (Fig. 1G).

### *3.2 Itaconic Acid, Citraconic Acid and Mesaconic Acid Can Reduce Renal Function Injury Caused by IRI*

In order to evaluate whether the intervention of itaconic acid, citraconic acid and mesaconic acid can effectively reduce renal impairment, biochemical analysis was performed on rat serum to detect the levels of Urea, Cr and Cys-C. Compared with the sham group, the serum Urea level of rats in the IRI group was increased ($p < 0.05$), while the serum Urea level of rats in the itaconic acid group ($p < 0.01$), citraconic acid group ($p < 0.001$) and mesaconic acid group ($p < 0.05$) was decreased compared with that of the IRI group (Fig. 2A). Compared with the sham group, Cr level in the IRI group was increased ($p < 0.05$), while Cr level in the itaconic acid group ($p < 0.05$), mesaconic acid group ($p < 0.05$) and citraconic acid group ($p < 0.001$) was decreased compared with the IRI group (Fig. 2B). The

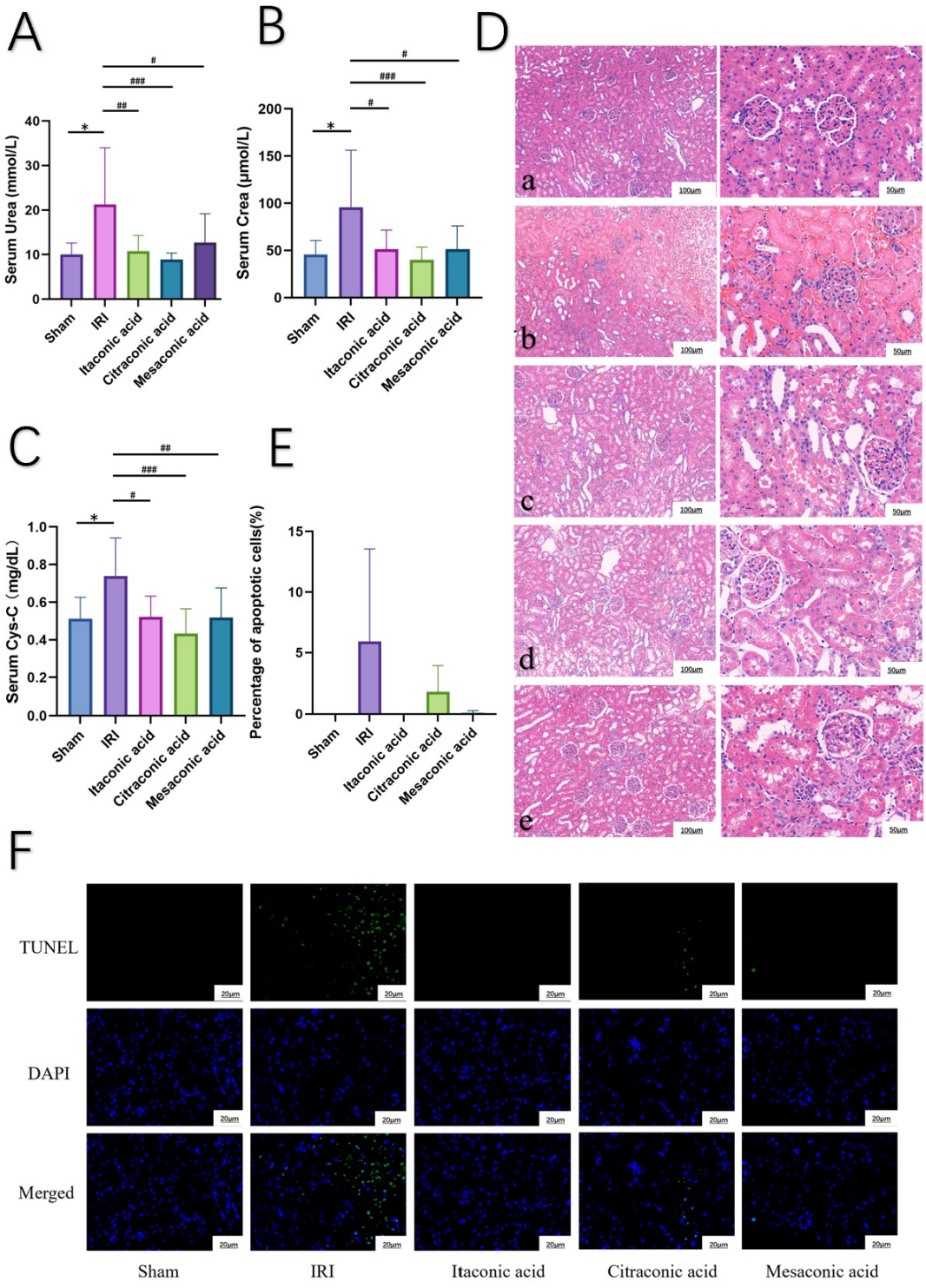



**Fig. 2. Itaconic acid, citraconic acid and mesaconic acid reduced renal function injury caused by IRI**. (A–C) Results of biochemical analysis of rat serum. Data were analyzed by non-parametric test, n = 10–13. Remarks: Compared with sham group: *$p < 0.05$; Compared with IRI group: #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$. (D) Renal pathology of rats represented by HE staining. Scale: left: 100 μm, right: 50 μm. (a) Sham Group; (b) IRI Group; (c) Itaconic acid group; (d) Citraconic acid group; (e) Mesaconic acid group. (E) Percentage of apoptotic cells in rat kidney. The data were analyzed by one-way ANOVA, n = 9 fields/groups. (F) Representative images of TUNEL staining of the rat kidney. Green: TUNEL, blue: DAPI. Scale: 20 μm. TUNEL, TdT-mediated dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

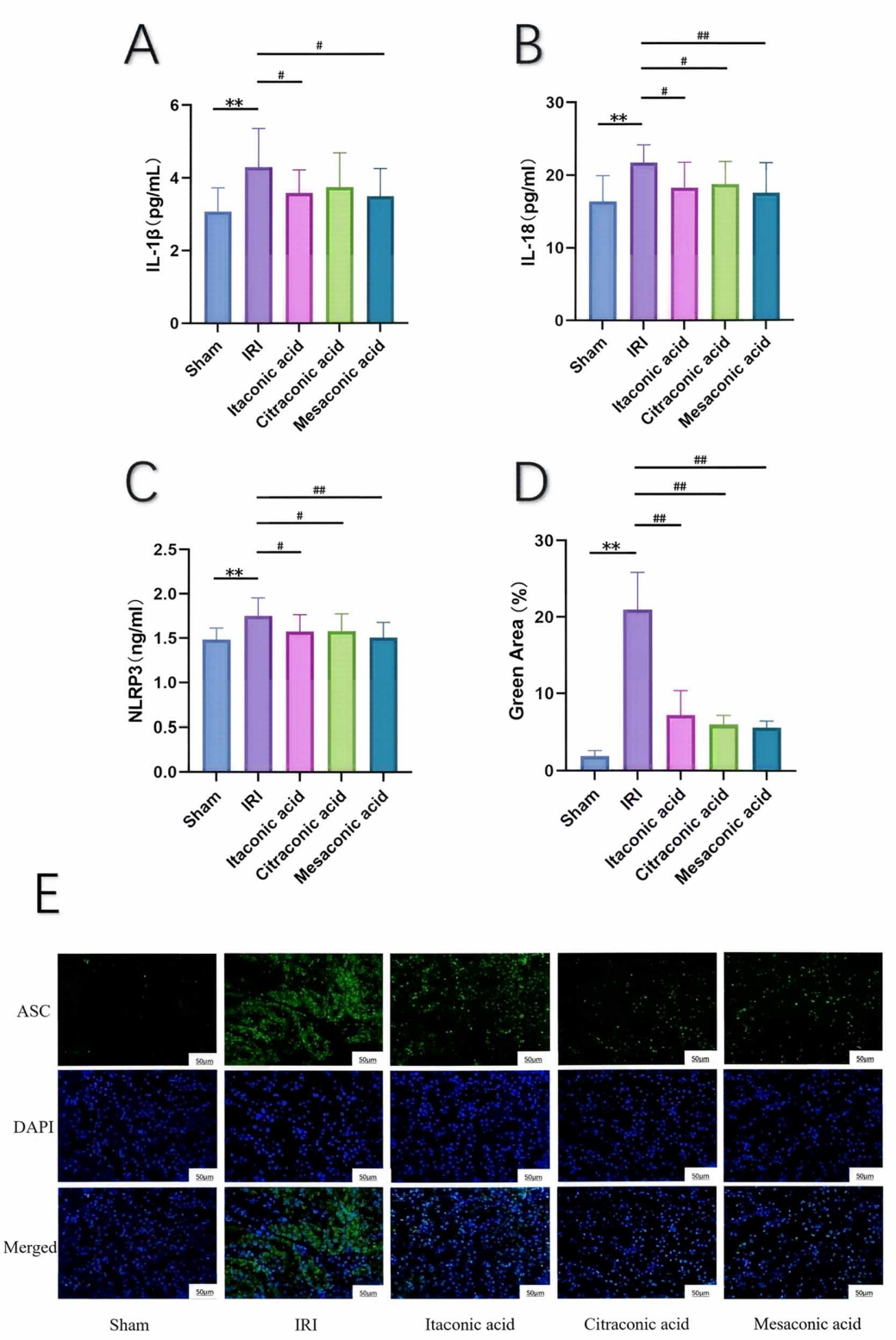



**Fig. 3. Itaconic acid, citraconic acid and mesaconic acid reduce IRI by reducing the activation of NLRP3 and the release of inflammatory cytokines**. (A–C) The levels of IL-1β, IL-18 and NLRP3 in the kidney of rats were analyzed by ELISA. Data were analyzed by one-way ANOVA, n = 10–13. (D) The proportion of green fluorescence-positive area in ASCimmunofluorescence staining of the kidney in rats. The data were analyzed by one-way ANOVA, n = 6 fields/groups. Remarks: Compared with sham group: $^{**}p < 0.01$; Compared with IRI group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. (E) Images represented by ASC immunofluorescence staining of rat kidneys. Green: ASC, blue: DAPI. Scale: 50 μm.

serum Cys-C level of rats in the IRI group was higher than that in the sham group ($p < 0.05$), while the serum Cys-C level in the itaconic acid group ($p < 0.05$), citraconic acid group ($p < 0.001$) and mesaconic acid group ($p < 0.01$) was lower than that in the IRI group (Fig. 2C). In order to further understand the degree of kidney injury in rats, HE staining was performed on the kidney tissues of rats in each group. The results showed that the renal tissue capsule in the sham group was relatively complete, and no connective tissue hyperplasia and inflammatory exudation were observed; the boundary between cortex and medulla was clear, and the renal corpuscle was complete and clear. The renal capsule cavity was clearly visible, and the capillary basement membrane or mesangial hyperplasia was not observed in the vascular globules. Renal tubular epithelial cell vacuolar degeneration, tubular epithelial cell degeneration and necrosis, tubular epithelial cell shedding, brush border shedding, and tubular formation were observed in the IRI group, and the renal tissue lesions in the itaconic acid group, citraconic acid group and mesaconic acid group were relatively mild (Fig. 2D). It was shown that renal function was obviously improved after drug intervention. However, TUNEL staining results showed that there were no significant differences in renal cell apoptosis among all groups ($p > 0.05$) (Fig. 2E,F). The results of biochemical analysis and pathological staining of rat kidneys showed that itaconic acid, citraconic acid and mesaconic acid could effectively reduce renal function injury caused by renal IRI.

### *3.3 Itaconic Acid, Citraconic Acid and Mesaconic Acid Reduce IRI by Reducing the Activation of NLRP3 and the Release of Inflammatory Cytokines*

4-OI has been shown to inhibit lactate dehydrogenase release, IL-18 release, ASC speck formation, and gasdermin D and IL-1βprocessing, all of which are indicators of NLRP3 activation [46]. To verify whether non-derived itaconic acid and its isomers have the same anti-inflammatory effects, we examined the levels of inflammatory cytokines IL-1β and IL-18 in rat kidneys. Compared with the sham operation group, IL-1β concentration in the kidney of the IRI group was significantly increased ($p < 0.01$). Compared with the IRI group, the renal IL-1β concentration in the itaconic acid group and mesaconic acid group was decreased ($p < 0.05$). There was no statistically significant difference in IL-1β concentration in the kidney of rats in the itaconic acid group (Fig. 3A). Compared with the sham group, IL-18 concentration in the kidney of the IRI group was increased ($p < 0.01$). IL-18 concentration in the kidney of rats in the itaconic acid group ($p < 0.05$), citraconic acid group ($p < 0.05$) and mesaconic acid group ($p < 0.01$) was lower than that in the IRI group (Fig. 3B).

We further tested the levels of the NLRP3 inflammasome in the rat kidneys using an ELISA kit. Compared with the sham group, NLRP3 concentration in the kidney of rats in the IRI group was significantly increased ($p < 0.01$). Compared with the IRI group, the concentration of NLRP3 in the kidney of rats in the itaconic acid group ($p < 0.05$), citraconic acid group ($p < 0.05$) and mesaconic acid group ($p < 0.01$) was decreased (Fig. 3C).

In addition, the frozen sections of rat kidney tissue were stained with ASC spot immunofluorescence, and the positive expression of ASC spots was green. The results showed that compared with the sham group, the expression of ASC spot protein in the renal IRI group was significantly increased ($p < 0.01$). The expression of ASC spot protein in the itaconic acid group, citraconic acid group and mesaconic acid group was significantly reduced compared with that in the renal IRI group ($p < 0.01$) (Fig. 3D,E).

### *3.4 Itaconic Acid, Citraconic Acid and Mesaconic Acid Can Reduce IRI-Induced AKI by Inhibiting Oxidative Stress*

Next, we measured the concentration of SDH in the rat kidney tissue. Compared with the sham group, SDH concentration in the kidneys of rats in the IRI group was decreased ($p < 0.01$). SDH concentration in the itaconic acid group and mesaconic acid group was increased ($p < 0.05$) compared with the IRI group. There was no statistical significance in SDH concentration in the kidney of rats in the citraconic acid group ($p > 0.05$) (Fig. 4A). The SDH in renal tissue of rats decreased after IRI. The addition of itaconic acid and mesaconic acid could increase the SDH level of rats, but citraconic acid had little effect on the SDH level.

We used DHE probes to measure ROS levels in the rats' kidneys. The results showed that compared with the sham group, the proportion of red fluorescence area in the tissue of rats in the IRI group was significantly increased, with statistically significant significance ($p < 0.01$). Compared with the IRI group, the proportion of red fluorescence area in renal tissue of rats in the itaconic acid, citraconic acid and mesaconic acid groups was significantly decreased ($p < 0.01$) (Fig. 4B,C). The results indicated that ROS concentration in renal tissues increased significantly after renal IRI, and ROS concentration in renal tissues of rats treated with itaconic acid, citraconic acid and mesaconic acid decreased significantly.

### *3.5 Itaconic Acid, Citraconic Acid and Mesaconic Acid Reduce IRIInduced AKI by Keap1-Nrf2 Axis*

In order to further study the effect of itaconic acid and its isomers citraconic acid and mesaconic acid on the renal IRI signaling pathway, we detected Nrf2 and Keap1 concentrations in rat kidneys. Compared with the sham operation group, the Nrf2 concentration in the IRI group was decreased ($p < 0.01$), while the Nrf2 concentration in the drug intervention group was significantly increased ($p < 0.05$) compared with the IRI group (Fig. 5A). Compared with the sham group, the Keap1 concentration in the kidneys of rats in the IRI group was increased ($p < 0.01$), while the

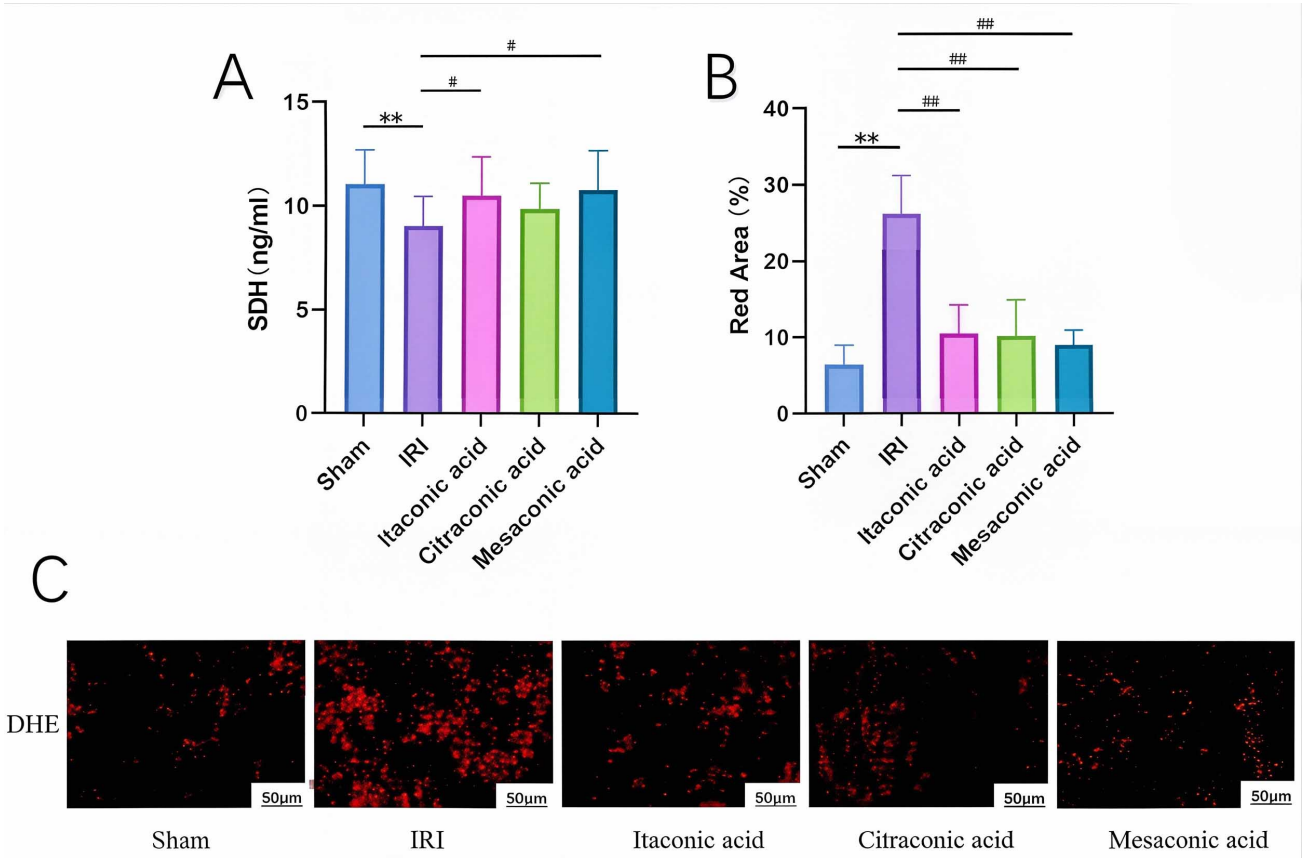



**Fig. 4. Itaconic acid, citraconic acid and mesaconic acid can reduce IRI-induced AKI by inhibiting oxidative stress.** (A) The changes in renal SDH concentration in each group were analyzed by ELISA. Data were analyzed by one-way ANOVA, n = 10–13. (B) The proportion of red fluorescence-positive area of kidney DHE staining in rats. The data were analyzed by one-way ANOVA, n = 6 fields/groups. Remarks: Compared with sham group: $^{**}p < 0.01$; Compared with IRI group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. (C) DHE fluorescent staining of rat kidney represents the image. Red: DHE. Scale: 50 μm.

Keap1 concentration in the itaconic acid group ($p < 0.01$), mesaconic acid group ($p < 0.01$) and citraconic acid group ($p < 0.05$) was decreased compared with the IRI group (Fig. 5B). These results indicate that itaconic acid, citraconic acid and mesaconic acid may reduce tissue damage by activating the Keap1-Nrf2 pathway.

## 4. Discussion

AKI is a syndrome with multiple etiologies that is characterized by rapid deterioration of kidney function occurring over a period of hours to days [4]. It is also a common complication of many critical diseases, with an incidence of about 13% to 73% [11]. Renal injury is affected by various pathological factors, and renal IRI is generally believed to be the basic pathogenesis of AKI. Ischemia-reperfusion is the temporary loss of blood flow and tissue perfusion followed by its restoration. Because blood flow stops, cells lack enough oxygen to synthesize ATP [47]. Renal IRI is closely related to ROS production, intracellular calcium overload, inflammation and apoptosis [48,49,50].

A key component of inflammation is the activation and recruitment of freely circulating white blood cells in the blood pool, resulting in impaired renal microcirculation and slowed blood flow in the renal cortex [51]. Ischemia and hypoxia in renal tissue lead to massive ROS release, and inflammatory cells release proinflammatory factors, recruiting large numbers of leukocytes. Microbubbles are engulfed by activated leukocytes, and the engulfed ones are still acoustically active, thus allowing the detection of microbubbles on ultrasound imaging [52]. The microbubbles exist in the inflammation area for a long time, resulting in slower clearance, and the Tp value increases, MTT prolongation, Peak value and AUC value increase, which are reflected in the TIC curve as a slow rise, and then a slow decline after reaching the peak.

The renal tissue inflammation in itaconic acid, citraconic acid and mesaconic acid groups was significantly alleviated, and the parameters showed a decrease in Tp value, a decrease in MTT value, and a decrease in Peak value and AUC value compared with the renal IRI group, which was reflected in the TIC curve showing a rapid rise in the curve and a rapid decline after reaching the peak value, basically consistent with the previous study results of Sun et al. [17]. CEUS can reflect the renal function status through changes

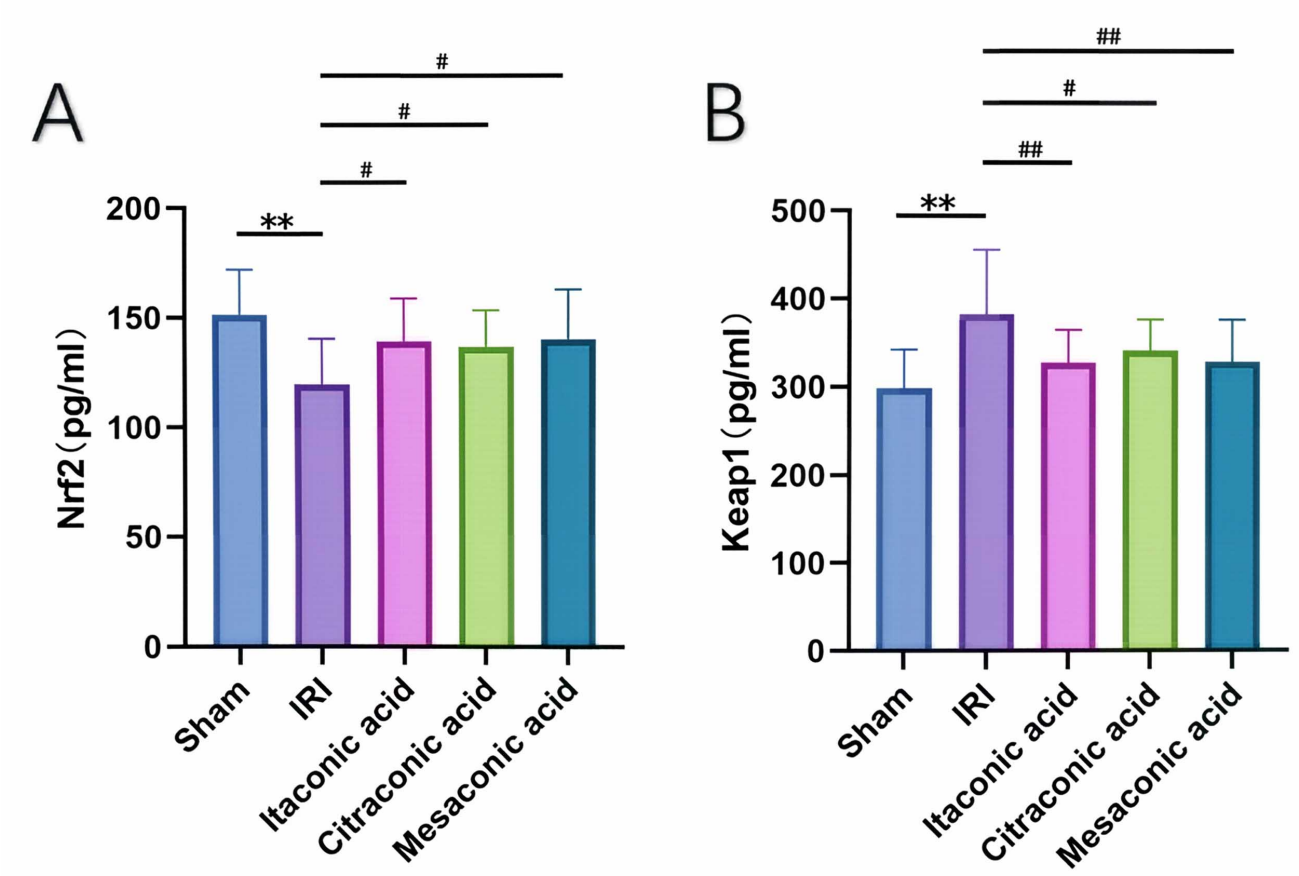



**Fig. 5. Itaconic acid, citraconic acid and mesaconic acid reduce IRI-induced AKI through Keap1-Nrf2 axis**. (A,B) The expression levels of Keap1 and Nrf2 in rat kidneys were analyzed by ELISA. Data were analyzed by one-way ANOVA, n = 10–13. Remarks: Compared with sham group: $^{**}p < 0.01$; Compared with IRI group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

in Tp, peak, AUC, MTT value and the TIC curve at the early stage of kidney, and the changes in the indicators are consistent with the pathological results, which indicates that we may timely detect the risk of renal IRI through early CEUS examination. At the same time, when CEUS was measured after drug treatment, all the indicators showed remission compared with those in the untreated group, indicating that CEUS can be used as an effective means to evaluate the efficacy of renal IRI in partial drug treatment. Therefore, CEUS can be used as a new non-invasive test to evaluate renal function [7].

In our experimental results, we detected the concentration of SDH in the kidney of rats, and the results showed that the concentration of SDH in the kidney of rats decreased after IRI, itaconic acid and mesaconic acid inhibited the activity of SDH, and the concentration of SDH in the kidney of rats increased compensatively, while citraconic acid almost did not increase the concentration of SDH. Itaconic acid is thought to limit inflammation by inhibiting SDH [53,54]. The similar structure of itaconic acid and succinic acid can directly competitively inhibit the enzymatic activity of SDH [37,55]. Previous studies have shown that itaconate can act as a mitochondrial regulator to control redox metabolism and improve cerebral IRI in mice. The main mechanism is to reduce tissue oxidative stress damage by inhibiting the activity of SDH and reducing ROS levels during reperfusion *in vivo* [56,57]. Itaconic acid and its isomers inhibit SDH activity to limit ROS production and reduce the release of inflammatory factors at the injury site [35]. *In vitro* studies have shown that itaconate has a strong inhibitory effect on SDH, while citraconate has no inhibitory effect on SDH, but it does reveal that mesaconate has a moderate inhibitory effect on SDH [45]. However, in the study of He et al. [58], it was believed that mesaconate had no inhibitory effect on SDH In our results, citraconic acid did not inhibit SDH, but still mitigated kidney damage, and we speculate that citraconic acid may inhibit oxidative stress through other pathways.

Itaconic acid can also inhibit inflammatory responses by blocking the assembly of inflammasomes and reducing the expression of NLRP3 [46]. This conclusion was also confirmed by an ELISA test of NLRP3, and citraconic acid and mesaconic acid also showed inhibitory effects on NLRP3. The NLRP3 inflammasome is composed of a variety of protein complexes, including the protein ASC. They assemble into inflammatory bodies in response to inflam-

matory stimulation, leading to tissue damage [59]. The experimental results showed that the exogenous addition of itaconic acid, citraconic acid and mesaconic acid all reduced the expression of ASC speck, inhibited the activation of the NLRP3 inflammasome, reduced the release of IL-18, and all of them except citraconic acid reduced the expression of the pro-inflammatory factor IL-1β, thus alleviating the acute kidney injury caused by renal IRI. NLRP3 inflammasome is a polymeric complex composed of a cytoplasmic sensor, caspase activation, ASC speck-like protein and pro-caspase-1. The inflammasome is stimulated by DAMPs and triggers inflammation by activating caspase-1. Caspase-1 cuts the N-terminal sequence of GSDMD, causing it to bind to the membrane to produce membrane pores, resulting in cell pyroptosis. Activated caspase-1 also promotes the release of inflammatory cytokines IL-1β and IL-18 [56,60,61], leading to interstitial immune cell infiltration and renal tubule injury [62]. It can be observed from the experimental results of this study that after renal IRI in rats, the expression of ASC spot protein is significantly increased, the activation of NLRP3 inflammation is increased, and the release of inflammatory factors IL-1β and IL-18 is increased. After treatment with itaconic acid and its isomers, the activation of the NLRP3 inflammasome and the release of inflammatory factors IL-1β and IL-18 were reduced in the intervention group. It can be seen that itaconic acid and its isomers can not only alleviate kidney injury by inhibiting oxidative stress, but also relieve renal IRI by inhibiting inflammation-related pyroptosis.

In our experimental study, the concentration of Keap1 in the kidney tissues of rats in itaconic acid, citraconic acid and mesaconic acid groups was significantly lower than that in the IRI group, while the concentration of Nrf2 in the kidney tissues of rats in itaconic acid, citraconic acid and mesaconic acid groups was significantly higher, which proved that not only itaconic acid could inhibit inflammation by activating Nrf2, but also citraconic acid and mesaconic acid, as isomers, can exert anti-inflammatory effects through the Keap1-Nrf2 pathway. Studies have shown that reduced Nrf2 levels are found in many kidney diseases with high levels of ROS [63]. This is consistent with our experimental results that Nrf2 can counteract ROS-mediated tissue damage [64]. Nrf2 is a major transcription factor in the inflammatory response and in controlling the antioxidant response that is necessary to maintain cellular redox homeostasis, and since oxidative stress is also a key driver of various kidney diseases, Nrf2 has been reported to prevent kidney disease by down-regulating the production of ROS [65]. Under normal conditions, Nrf2, in combination with Keap1, promotes its ubiquitination and proteasomal degradation. Itaconic acid directly modifies proteins through the alkylation of cysteine residues, leading to the conformational change of the Nrf2-Keap1 complex, inhibiting the degradation of Nrf2, and increasing the expression of Nrf2, which has antioxidant and anti-inflammatory capabilities [53]. Studies have shown that citraconic acid is the strongest electrophile among the three isomers, resulting in the strongest activation of Nrf2 [45]. In our experimental results, we did not compare the differences in the activation of Nrf2 by the three isomers, which may be related to the differences in membrane permeability and cell absorption of the three substances, and the specific reasons for the differences need to be further studied.

In addition to its appeal mechanism, itaconic acid induces the activation of activating transcription factor 3, which participates in the body's anti-inflammatory effects [53,66,67]. Besides, itaconic acid selectively inhibits dioxygenase to inhibit inflammation [68]. Itaconic acid also plays an important anti-inflammatory role as a glycolysis inhibitor [69]. These pathways could not be verified in our study, but it cannot be ruled out that they also play a key role in reducing renal IRI. At present, itaconic acid has shown good therapeutic effects in many animal disease models such as sepsis and pulmonary fibrosis [70,71,72,73,74,75]. Itaconic acid also has great potential research value in regulating central nervous system diseases [76,77]. Various itaconic acid derivatives such as 4-OI and dimethyl itaconic acid exhibit similar biological properties [78,79,80]. Fumaric acid, which has a similar structure to itaconic acid, is already used clinically to treat multiple sclerosis and psoriasis. In our study, the isomers of itaconic acid and fumaric acid were used as experimental drugs to intervene in renal IRI, and it was found that they were also feasible in the treatment of acute kidney injury, which proved that itaconic acid and its various derivatives and isomers have great research value and application prospects in the field of disease treatment.

The innovation of this study: First, fumaric acid, which is similar in structure to itaconic acid, has been applied in clinical treatment, and itaconic acid and its derivatives also have similar biological properties to fumaric acid. As a popular drug in preclinical studies on pharmacological intervention and pathological models, itaconic acid has demonstrated great application value in a variety of pathological models. It has a promising clinical translational prospect as a therapeutic strategy to reduce IRI. Second, this study also studied the intervention effect of citraconic acid and mesaconic acid, isomers of itaconic acid, on IRI. Citraconic acid and mesaconic acid have anti-inflammatory and antioxidant effects similar to itaconic acid, but at present, there are few data on the application of these two substances in experimental studies. This study is the first to apply these two substances in the model of treating AKI. The experimental data on citraconic acid and mesaconic acid in the treatment of these diseases are relatively lacking, and provide more possible new strategies for the treatment of IRI-induced AKI. Third, CEUS technology was used in this study to monitor blood perfusion of renal IRI in real time, and quantitative TIC parameters (Peak, Tp, AUC, MTT) of CEUS technology were compared with biochemical indica-

tors and pathological results reflecting renal function status. We found that CEUS can reflect the AKI condition of the kidney after IRI in an early, accurate and effective manner. The combination of CEUS results with AKI biomarkers and pathological results can provide a new means for the early diagnosis and prognosis assessment of ischemia-reperfusion-induced AKI.

*Limitations*

Limitations of this experiment: It is important to acknowledge the limitations of the pharmacological data presented in this study. Despite the findings presented in this study, several limitations should be acknowledged. First, the sample size of the animal experiment was relatively small, which might limit the statistical power. Future studies with larger cohorts are warranted to validate these observations. Second, the animal research model of this experiment can be further refined. This time, only male rats were selected, and the impact of gender differences was not compared. Previous studies have shown that gender may express varying degrees of sensitivity to AKI. In our subsequent research, we will consider including comparisons between different gender groups. Third, only a one-time point of 24 hours was selected for detection in this study, and multiple time points could be selected for dynamic detection of rat kidney changes in subsequent experiments, to obtain the data results of long-term prognosis. Fourth, while this study investigates the similar anti-inflammatory and antioxidant mechanisms of itaconic acid, citraconic acid and mesaconic acid, we cannot exclude the possibility that other signaling pathways may also be involved in the observed effects. However, more precise molecular mechanisms still require further research through more technical means.

## 5. Conclusions

Itaconic acid and its isomers citraconic acid and mesaconic acid have similar inhibitory effects on inflammation, improve the antioxidant capacity of renal tissues, and may alleviate renal injury caused by IRI through inhibiting NLRP3 inflammasome-associated inflammation, providing a new strategy for the treatment of AKI. CEUS can effectively evaluate the renal cortical blood perfusion of renal IRI, and CEUS can quantitatively evaluate the changes in renal function after drug intervention, providing an effective examination method for clinical diagnosis, treatment and prognosis assessment of this disease.

## Availability of Data and Materials

All raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

## Author Contributions

BT participated in the topic selection, article writing and illustrations. ZL participated in the topic selection, article modification, and put forward many valuable guidelines. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

All animal experiments were conducted in strict accordance with the guidelines of the Affiliated Hospital of Southwest Medical University Animal Care and Use Committee. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of the Southwest Medical University (2024100045). All authors consent to the publication of this paper. All animal experimental procedures were conducted in accordance with the principles of the 3Rs (Replacement, Reduction, and Refinement), and the study was reported following the ARRIVE guidelines for animal research.


## Acknowledgment

Not applicable.

## Funding

This research was supported financially by Major Cultivation Project for the Bureau of Science and Technology of Nanchuan district of Chongqing city (No.Nckjcx20240101); Open Project of Sichuan Provincial Clinical Medical Research Center for Birth Defects (2019YFS0531-6).


## Conflicts of Interest

The authors declare no conflicts of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL52184.

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