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Original Research Article

Erdosteine prevents contrast-induced renal oxidative stress damage in mice

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Abstract

Purpose: *To investigate the protective effect of erdosteine on contrast-induced renal oxidative stress in mice.*
Methods: *C57BL/6 mice were injected intraperitoneally with contrast medium to establish an acute kidney injury model (AKI). Renal function, blood urea nitrogen (BUN) and serum creatinine (SCr) were determined. Also, oxidative stress, lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) were evaluated. And the renal tissue structure was examined by light microscopy, while Western blot (WB) and Real-time polymerase chain reaction (RT-PCR) were used to determine the expressions of senescence-related factor, Nrf-2 and downstream antioxidant factor.*
Results: *Erdosteine improved the renal structure of mice and significantly decreased serum BUN and SCr levels. In addition, erdosteine promoted the expression of antioxidant enzymes SOD1, SOD2, GPX1 and GPX3 in renal tissues, decreased the content of ROS, and inhibited the content of LDH and MDA in serum. Also, WB and RT-PCR results showed that erdosteine activated Nrf2 pathway, thereby alleviating contrast-induced renal injury.*
Conclusion: *Erdosteine inhibits contrast-induced renal oxidative stress in mice and delays cell senescence by activating Nrf2 pathway. This will be of great significance in the treatment of contrast-induced nephropathy.*

Keywords: *Erdosteine, Contrast agent, Nrf-2, HO-1, Oxidative stress*

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INTRODUCTION

Contrast-induced acute kidney injury (CI-AKI) is a major complication of various iodine-contrast related diagnostic measures and angiography techniques [1]. Although the probability of developing CI-AKI is relatively low in patients with normal renal function, the incidence of CI-AKI is significantly high in patients with chronic kidney disease, reaching 27 %. CI-AKI is defined as the absolute value of serum creatinine (SCr) increased ≥ 0.05 mg/ dL (44.2 μmol/L) or increased ≥ 25% relative to the baseline value after 48 ~ 72 h of contrast agent exposure, and other factors that may cause renal damage were excluded [2]. CI-AKI seriously impairs renal function, increases the risk of related

complications, and leads to prolonged hospitalization, which imposes a huge economic burden on the patient's family and society. Numerous studies have confirmed that the main mechanisms of contrast-induced renal injury are vasoconstriction and renal ischemic injury, direct toxicity of contrast agents to renal tubular cells, oxidative stress hemodynamic changes and inflammatory response [3]. This study focused on the protective effects of erdosteine against contrast-induced renal oxidative stress.

Erdosteine is an expectorant that has been used clinically. A large number of experimental studies have confirmed that its metabolites of liver biotransformation also have the functions of scavenging free radicals, anti-oxidative stress injury, and protecting vital organs from ischemia/reperfusion injury [4]. However, the role of erdosteine in CI-AKI has not been studied. Nrf2 is a major regulator of ARE mediated gene expression and a receptor for exogenous toxic substances and oxidative stress [5]. Normally, Kelch-like epichlorethane associated protein-1 (KEAP1) collects Nrf2 in the cytoplasm along with the actin cytoskeleton. When attacked by reactive oxygen species or nucleophilic agents, Nrf2 dissociates from KEAP1, enters the nucleus and binds to ARE, thereby activating transcription of several antioxidant genes such as superoxide dismutase, γ-glutathione synthase, and heme oxygenase-1 (HO-1) [6]. Whether Erdostan can effectively reduce contrast agent induced oxidative stress injury through activation of Nrf2/ HO-1 signaling pathway has not been reported yet.

EXPERIMENTAL

Laboratory animals and drug preparation

Eight-week-old wild-type male C57BL/6 mice were procured from the animal center of PLA Medical School. Mice were housed in animal centers with an average temperature of 20 ± 2 °C, relative humidity of 50 - 70 %, and day and night lighting for 12 h /12 h. The animal experiments were strictly according to the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. This study was approved by the Animal Ethics Committee of PLA Medical School Animal Center (13-CN-BJ-PMSEC-3421). All procedures were conducted in accordance with 'Animal Research: Reporting in Vivo Experiments guidelines 2.0' [7]. Erdosteine was dissolved in phosphate buffered saline (PBS) and stored at -20 ℃ afterwards. Working solution was prepared with Dulbecco's modified eagle medium (DMEM) before use.

Collection of samples

The mice were weighed, and sacrificed with intraperitoneal injection. Abdominal aorta blood was collected, and the blood sample was coagulated naturally. The blood sample was centrifuged at 3000 rpm/min for 10 min, and the supernatant was kept at -80 °C. Both renal tissues were separated and rinsed with PBS. The kidneys were rapidly frozen in liquid nitrogen and stored at -80 °C prior to subsequent analysis.

Determination of biochemical indicators

The blood of each group was collected, and the serum was collected after centrifugation. The contents of blood urea nitrogen (BUN), serum creatinine (SCr), malondialdehyde (MDA) and dehydrogenase (LDH) in each group were determined according to the instructions of BUN, SCr, MDA and LDH assay kits (Jiangcheng, Nanjing, China).

Hematoxylin-eosin staining

Mouse kidney tissue was fixed with 10 % paraformaldehyde, and tissue was fixed with paraffin (Yunqing, Changzhou, China). The embedded blocks were cut into thin slices (5 μm thick) with a slicer and observed under a microscope (Sino, Beijing, China). The sections were dewaxed with xylene, fractionated with ethanol and rehydrated, stained with sappanwood semen for 5min and then stained with 0.5% eosin for 2min. The morphological changes in kidney injury were examined under a microscope after sealing with a sealer.

Immunofluorescence

After dewaxing the sections, the endogenous peroxidase was inactivated with a 3% H_2O_2 solution, washed 3 times, and then blocked with 10% goat serum for 1 h. Then these sections were incubated overnight at 4 °C with diluted primary antibody (SOD1, 1:1000, Abcam, Cambridge, MA, USA). The next day, after rinsing the sections, the diluted secondary antibody was incubated in the dark for 1 h. Finally, after staining the nuclei with 4'6-diamidino-2-phenylindole (DAPI), the sections were covered and observed under a fluorescence microscope.

Flow cytometry

The kidney tissue was separated, placed in 1 mL of pre-cooled PBS liquid and cut. Renal tissue cells were filtered through a 70 μm screen to adjust the cell concentration. The prepared 1 mL

single-cell suspension was added to 2 ', 7 '-dichlorofluorescein diacetate (DCF-DA) for 5 μl incubation, the supernatant was removed by centrifugation, and incubated with 10% fetal bovine serum. After centrifugation at 4°C, a single cell suspension of kidney tissue was prepared. Flow cytometry (BD FACSC alibur type, Becton-Dickinson (BD), Franklin Lakes, NJ, USA) was used to measure the average fluorescence intensity of intracellular marker fluorescent probes.

Enzyme-linked immunosorbent assay (ELISA)

PBS was added to an appropriate amount of kidney tissue, and the supernatant was collected by homogenization and centrifugation for 10 min. Standard wells and sample wells were set, and standard substance of different concentrations was added to each standard well. Horseradish peroxidase (HRP) labeled detection antibody was added to each standard well and sample well, and incubated in the incubator for 1 h. After the liquid was discarded, working solution (Jianglai, Shanghai, China) was added to each well and incubated for 15 min in the dark. Finally, the absorbance (A) of each well was measured at a wavelength of 450 nm.

Western blot

The appropriate amount of kidney tissue of mice in each group was taken to extract kidney tissue protein, and the protein concentration of the sample was determined by bicinchoninic acid (BCA) protein concentration determination kit (Pierce, Rockford, IL, USA). An equal amount of protein sample was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5 % skim milk for 2 h, the membranes were incubated with primary antibodies overnight at 4 °C. The next day, the corresponding secondary antibody was incubated at room temperature, which was observed by an electrochemiluminescence (ECL) system. The absorbance of the target bands was analyzed by using AlphaEasefc software processing system. Specific antibody: SOD1 (1:2000, Abcam, Cambridge, MA, USA, Rabbit), SOD2 (1:2000, Abcam, Cambridge, MA, USA, Rabbit), GPX1 (1:3000, Abcam, Cambridge, MA, USA, Rabbit), GXP3 (1:3000, Abcam, Cambridge, MA, USA, Rabbit), Sirt1 (1:2000, Abcam, Cambridge, MA, USA, Mouse),Bmi-1 (1:1000, Abcam, Cambridge, MA, USA, Mouse), P53 (1:2000, Abcam, Cambridge, MA, USA, Mouse), P27 (1:2000, Abcam, Cambridge, MA, USA, Mouse), Nrf2 (1:1000, Abcam, Cambridge, MA, USA, Mouse), NQO-1 (1:1000, Abcam, Cambridge, MA, USA, Mouse), Txnrd-1 (1:1000, Abcam, Cambridge, MA, USA, Mouse), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (US 1:1000 CST, Danvers, MA, USA).

Real-time polymerase chain reaction (RT-PCR)

Each group of kidney tissues was washed with pre-cooled PBS and then total RNA was extracted by Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription and polymerase chain reaction were carried out, and the reaction conditions were: pre-deformation, 95°C, 1 min, 95 °C, 15 s, 58 °C, 20 s, 72 °C, 45 s, 40 cycles. Using GAPDH as an internal reference, the corresponding ΔΔCt values in each group of cells were calculated, and the quantitative analysis was performed based on the quantitative amount of the target factor ($2^{-\Delta\Delta Ct}$). The RT-PCR primers used are shown in Table 1.

Table 1: RT-PCR primers

Gene name	Forward (5'>3')	Reverse (5'>3')
HO-1	GTGACAGAAGAGGCTAAGACCG	CAACAGGAAGCTGAGAGTCAGG
NQO-1	GAGAAGAGCCCTGATTGTACTGC	GATGACTCGGAAGGATACTGAAAG
Txnrd1	CTCGGACAAGCTCTGAAAGACTC	GATGAGGAACCGCTCTGCTG
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
GPX1	ATCATATGTGTGCTGCTCGGCTAGC	TACTCGAGGGCACAGCTGGGCCCTTGAG
GPX3	AGAGCCGGGGACAAGAGAA	ATTTGCCAGCATACTGCTTGA
Sirt1	CCAGATCCTCAAGCCATG	TTGGATTCCTGCAACCTG
Bmi-1	ATCCCCACTTAATGTGTGTCCT	CTTGCTGGTCTCCAAGTAACG
P53	CTCTCCCCCGCAAAAGAAAAA	CGGAACATCTCGAAGCGTTTA
P27	GAGGGCAAGTACGAGTGCCAA	CTGCGCATTGCTGGCCTAACC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR = quantitative reverse-transcription polymerase chain reaction

Statistical analysis

All data were processed using SPSS 17.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). The data obtained are expressed as mean ± SD (standard deviation). Comparison between multiple groups was done using One-way ANOVA test followed by post hoc test (least significant difference). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Erdosteine ameliorates renal structural changes and functional impairment

H & E staining, the glomeruli of the control group were normal, and the renal tubules showed no edema, while the glomerular size of the contrast group was significantly changed, the tubule edema was obvious, and the interstitial fibrosis was severe. Erdosteine treatment significantly attenuated renal structural changes caused by contrast agents (Figures 1 A and B). The results showed that the levels of SCr and BUN in the contrast agent group were significantly increased compared with control group. However, the contents of SCr and BUN in the erdosteine group were significantly decreased when compared with the contrast agent group (Figures 1 C and D).

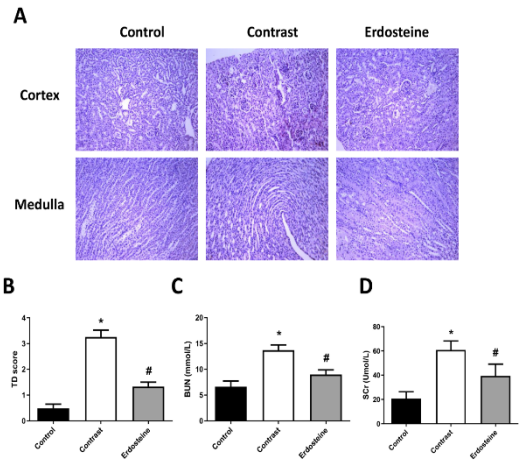

Figure 1: Erdosteine ameliorates renal structural changes and functional impairment. (A) and (B) H & E staining of mouse renal tissue (magnification:200x) and renal TD analysis. (C) and (D) Serum SCr and BUN levels. "*" Indicates statistical difference from the control group, $p < 0.05$; "#" indicates statistical difference from the contrast group, $p < 0.05$

Erdosteine relieves renal redox imbalance

First of all, WB and RT-PCR showed that the expression of SOD1, SOD2, GPX1 and GPX3 were significantly inhibited in the contrast agent group compared with the control group. (Figure 2 A - 2 F). Compared with the contrast agent group, Erdosteine treatment can effectively promote SOD1, SOD2, GPX1 and GPX3 expression. In addition, SOD and GSH levels were determined by using ELISA assay. The result showed that Erdosteine treatment can effectively inhibit the decrease of SOD and GSH content caused by contrast agents (Figure 2 G and H).

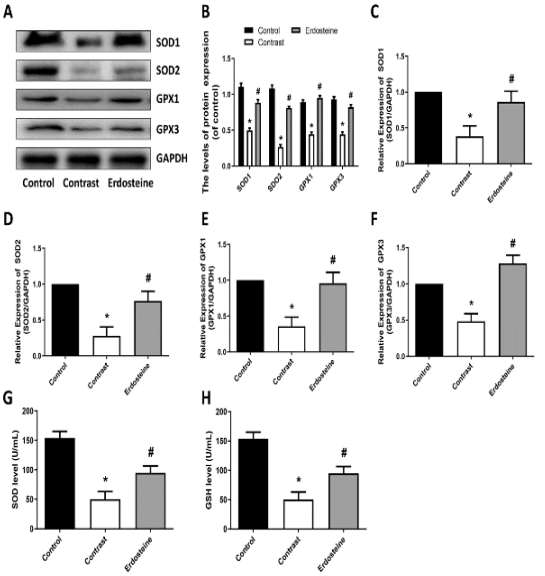

Figure 2: Erdosteine ameliorates renal redox imbalance. (A) and (B) Representative WB and quantified data show expressions of SOD1, SOD2, GPX1 and GPX3. (C~F) RT-PCR detection of SOD1, SOD2, GPX1 and GPX3 mRNA expression levels. (G) and (H) Renal tissue SOD and GSH content. "*" indicates statistical difference from the control group, $p<0.05$; "#" indicates statistical difference from the contrast group, $p < 0.05$

Erdosteine inhibits renal oxidative stress damage

Using immunofluorescence assay to determine the expression of SOD1, the result showed that compared with the control group, the total SOD1 fluorescence was significantly decreased in the contrast medium group. However, erdosteine treatment effectively promotes the total amount of SOD1 fluorescence (Figure 3 A). The LDH and MDA levels were increased in the contrast group and significantly decreased in the Erdosteine group (Figure 3 B and C). Secondly, flow cytometry results also confirmed that ROS were remarkably elevated in the contrast group, while Erdosteine was effective in inhibiting ROS elevation (Figure 3 D).

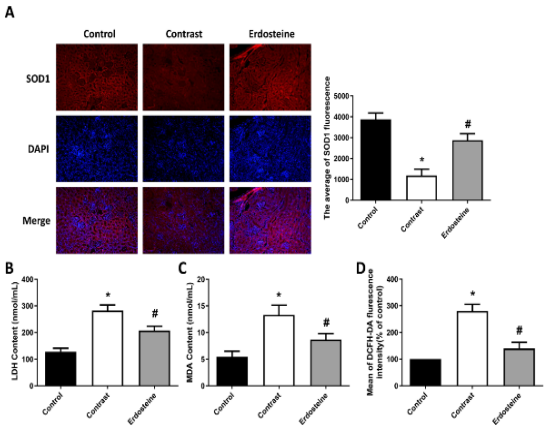

Figure 3: Erdosteine inhibits renal oxidative stress damage. (A) Immunofluorescence was used to determine tissue SOD1 expression (magnification: 200×). (B) and (C) Serum LDH and MDA content. (D) Renal tissue ROS content. "*" indicates statistical difference from the control group, $p < 0.05$; "#" indicates statistical difference from the contrast group, $p < 0.05$

Erdosteine inhibits kidney cell senescence

WB (Figures 4 A and B) and RT-PCR (Figures 4 C ~ F) results illustrated that kidney cells showed a significant senescence trend after contrast agent treatment. Sirt1 and Bmi-1 expressions were significantly inhibited in the contrast group, while P27 and P53 expressions were significantly increased. By contrast, in the Erdosteine group, Sirt1 and Bmi-1 expressions were significantly promoted, while P27 and P53 expression were significantly inhibited.

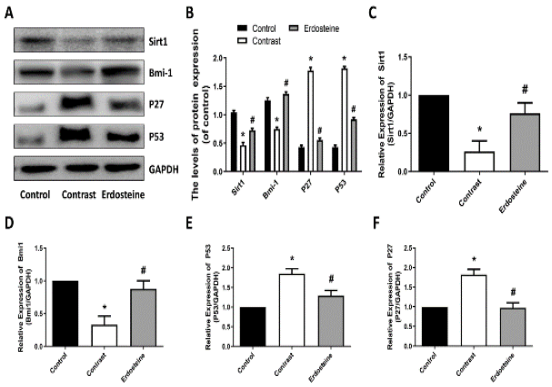

Figure 4: Erdosteine inhibit kidney cell senescence. (A) and (B) Representative WB and quantified data showed expressions of Sirt1, Bmi-1, P27, P53. (C~F) RT-PCR detection of Sirt1, Bmi-1, P27, P53 mRNA expression levels. * indicates statistical difference from the control group, $p < 0.05$; "#" indicates statistical difference from the contrast group, $p < 0.05$

Erdosteine activates Nrf2/HO-1 signaling pathway

Nrf2/ HO-1 signaling pathway plays an important role in the AKI development. Western blotting showed that contrast agent treatment could inhibit the expression of Nrf2 and its downstream proteins NQO-1 and TXNRD-1, while Erdosteine could promote the expression of Nrf2, NQO-1 and TXNRD-1. (Figure 5 A and B). Real-time PCR results showed that Erdosteine treatment can effectively inhibit the decrease of HO-1, NQO-1 and Txnrd-1 mRNA expression induced by contrast agents (Figure 5 C - E). And ELISA assay obtained similar results (Figure 5 F and G). Thus, all results showed that Erdosteine can activate the Nrf2/HO-1 signaling pathway.

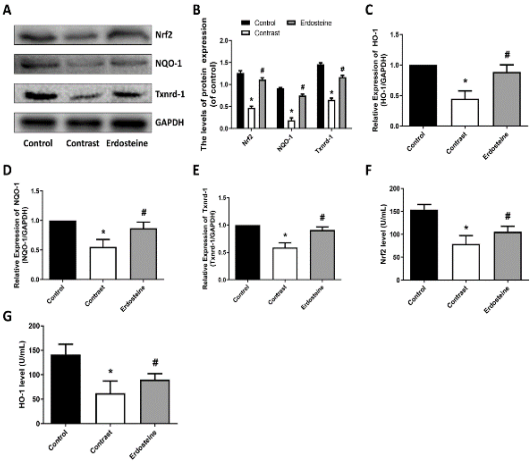

Figure 5: Erdosteine activates Nrf2/HO-1 signaling pathway. (A) and (B) Representative WB and quantified data show expressions of Nrf2, NQO-1, Txnrd-1. (C~E) RT-PCR detection of HO-1, NQO-1. Txnrd-1 mRNA expression levels. (F) and (G) ELISA detection of Nrf2, HO-1 expressions. ("*" indicates statistical difference from the control group, $p < 0.05$; # indicates statistical difference from the contrast group, $p < 0.05$

DISCUSSION

With the popularity of imaging and interventional therapy, the frequency of use of angiography in clinical practice is constantly on the increase, as well as the incidence of CI-AKI, which has become the third major cause of hospital-acquired acute renal failure [8]. Among the cardiovascular diseases, the associated mortality rate caused by CI-AKIis 34% [9]. The occurrence of CI-AKI not only prolongs the hospitalization days and increases the cost of treatment, it also causes irreversible renal damage and high risk of death [10]. Although the underlying pathological mechanism of CIN is still unclear, contrast

agents make renal perfusion reduction play a significant role in the development of CIN [11].

Erdosteine hasanti-oxidative stress effect. Numerous studies have reported that erdosteine has an antioxidant effect on ovarian ischemia-reperfusion injury [12]. In addition, Tutanc M found a protective effect of Erdosteine on cyclosporine-induced nephrotoxicity in rats [13]. However, erdosteine's mechanism of action on CI-AKI has not been reported. Then, we established a model of ARF by tail vein injection of iodixanol injection to study the corresponding mechanism.

The main causes of CI-AKI were 1) vasoconstriction and renal ischemia injury, 2) direct toxicity of contrast agents to renal tubule cells, 3) oxidative stress, 4) hemodynamic changes, and 5) inflammatory reaction. Jeong *et al* [14] found that oxidative stress promotes contrast-induced AKI. Previous studies have found that fasudil prevents contrast-induced AKI [15]. In this study, the results showed that Erdosteine also has a protective effect on the kidneys, which has similar effects to fasudil. Therefore, this paper mainly focused on the protective effect of Erdosteine on contrast agent induced oxidative stress.

The results showed that the kidney structure of the mice treated with contrast agent was significantly disordered, the glomerular size was different, the renal tubules were obviously edematous, and renal function was significantly reduced. At the same time, Western blot and Real-time PCR that the oxidative stress response of the contrast agent group increased significantly, leading to the decrease of antioxidant factor expression. At the same time, the results showed that the expression of SOD1 was also significantly inhibited by immuno-fluorescence. In contrast, erdosteine treatment is effective in promoting the expression of the antioxidant factors, thereby alleviating renal redox imbalance caused by contrast agents. In addition, we tested the senescence-related factors. The results confirmed that contrast agent treatment accelerated renal cell senescence: P27 and P53 expression were significantly increased, Sirt1 and Bmi1 expression were effectively inhibited, but erdosteine effectively inhibited cell senescence.

Nrf2 is expressed in the heart, liver, kidney and other organs, has anti-oxidative stress damage, and maintains the role of cellular redox homeostasis [16]. Nrf2 has many antioxidant factors downstream, including: SOD, HO-1, NQO-1 and Txnrd-1. Nrf2 is also involved in the GSH redox system [17]. Western blot results showed that Nrf2 expression was remarkably elevated in the erdosteine group when compared with contrast agent group. SOD and HO-1 are distributed in the cytoplasm and mitochondria of the cells, and the superoxide radicals are converted into water by disproportionation reaction, thereby exerting an antioxidant effect [18]. The results confirmed that contrast agent treatment significantly inhibited the expression of SOD and HO-1, but erdosteine inhibited the decrease of SOD and HO-1. NQO-1 and Txnrd-1 provide electrons through NADH and NADPH, which catalyze the reduction of steroids and their derivatives, causing their toxic degradation to exert antioxidant effects [19]. In this study, erdosteine activated Nrf2, which induce the expression of downstream NQO-1 and Txnrd-1 to exert an antioxidant effect and protect against contrast-induced AKI.

Erdosteine inhibited contrast-induced renal oxidative stress damage by activating the Nrf2/HO-1 signaling pathway, thereby inhibiting cellular senescence. Therefore, the Nrf2/HO-1 signaling pathway is considered to be a new therapeutic target for CI-AKI.

CONCLUSION

Erdosteine inhibits contrast-induced renal oxidative stress damage by activating Nrf2/HO-1 signaling pathway, thereby suppressing cellular senescence. Therefore, Erdosteine is a potential therapeutic agent for the prevention and treatment of CI-AKI.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qiwei Zhou and Feng Qian contributed equally to this work.

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