

Chrysin protects against renal ischemia reperfusion induced tubular cell apoptosis and inflammation in mice

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Abstract. Renal ischemia reperfusion (IR) is a major cause of acute kidney injury with no effective treatment. Chrysin is an anti-inflammatory, anti-oxidant and anti-cancer agent. However, the effect of chrysin on renal IR injury remains unknown. In this study, sham operation, IR and IR+chrysin group mice were treated with or without renal IR injury. For renal IR, bilateral renal pedicles were clamped for 30 min and then released for 48 h of reperfusion. Blood and kidney samples were collected for analysis. Results demonstrated that chrysin pretreatment remarkably decreased the levels of serum creatinine and blood urea nitrogen and attenuated morphological abnormalities in renal IR injury. Consistently, tubular cell apoptosis and inflammation were more attenuated in the chrysin pretreatment group compared with the IR group. Chrysin pretreatment decreased the expression of Bax and cleaved caspase-3 and increased the expression of Bcl-2 in renal IR injury. Furthermore, chrysin administration decreased the mRNA and protein levels of tumor necrosis factor- α , interleukin (IL)-1 β , and IL-6. Furthermore, the I κ B α /nuclear factor- κ B signaling pathway was more suppressed in the chrysin pretreatment group compared with the IR group. In conclusion, chrysin protects against tubular cell apoptosis and inflammation in renal IR injury.

Introduction

Renal ischemia reperfusion (IR) injury is a common cause of acute kidney injury (AKI) (1) and characterized by high morbidity and mortality (2). In clinical settings, patients subjected to kidney transplantation and renal tumor resection inevitably suffer from renal IR injury (3). Renal tubular cell

apoptosis and inflammatory response are the most important pathophysiological process of ischemic AKI (4). Following IR, the tubular cells in the outer medulla suffer the most severe injury, leading to renal dysfunctions (5). In addition, inflammatory response promotes renal dysfunctions and progressive chronic kidney disease (6). Therefore, inhibiting tubular cell apoptosis and inflammatory response may be an effective treatment of renal IR injury.

Chrysin is a naturally occurring flavonoid with anti-inflammatory, anti-oxidant and anti-cancer properties (7). It ameliorates indomethacin-induced inflammatory response and oxidative injury (8), and suppresses tumor growth of murine melanoma (9). Additionally, it attenuates focal cerebral IR injury in mice (10). However, its effect on renal IR injury remains unknown.

In this study, a renal IR injury model was established in mice and the effects of chrysin on renal IR injury were investigated. Results demonstrated that chrysin remarkably attenuated IR-induced renal dysfunctions and morphological abnormalities. Furthermore, chrysin inhibited renal IR-induced tubular cell apoptosis and inflammatory response. Therefore, chrysin may protect against renal IR-induced ischemic AKI.

Materials and methods

Animals and treatment. All experiments were approved by the Institutional Animal Care and Use Committee at Hubei University of Arts and Science (Xiangyang, China). The surgical procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 8023, revised 1978). A total of 30 male C57BL/6 mice (8-10 weeks old) were purchased from the Center of Experimental Animals of Wuhan University (Wuhan, China) and housed in a humidity (50-60%) and temperature-controlled environment with a 12-h light/dark cycle and free access to food and water. Mice were randomly divided into three groups (each, n=10): Sham, IR and IR+chrysin. To induce renal IR injury *in vivo*, the mice were abdominally anesthetized with phenobarbital sodium (60 mg/kg) and their body temperature was maintained at 37°C. Flank incisions were also conducted to expose the pedicels. The IR and IR+chrysin group mice were subjected to bilateral renal pedicle clamping for 30 min and reperfusion for 48 h. The sham group mice only underwent exposed pedicles

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without pedicle clamping and received injections of an equal volume of saline. Blood and kidney samples were collected for analysis. Chrysin was purchased from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany (95082) and IR+chrysin group mice were injected with chrysin for 3 days (100 mg/kg each time) prior to IR operation.

Renal function assay. The blood (200 μ l) was collected and centrifuged (3,500 x g) at 4°C for 15 min. Thereafter, the supernatant was harvested and stored at -80°C. The serum concentrations of creatinine (Cr) and blood urea nitrogen (BUN) were tested using creatinine and urea assay kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) in accordance with the manufacturer's protocol.

Hematoxylin and eosin (H&E) assay. To evaluate kidney injury score, renal samples were fixed in 4% formaldehyde at room temperature for 24 h, embedded in paraffin and cut into 4 μ m sections, stained with hematoxylin (8 min), and eosin (2 min) at room temperature. Histological features were imaged using a light microscope (Olympus Corporation, Tokyo, Japan) and a total of 10 random fields of view were obtained from the cortico-medullary region. For assessment of renal injury score, tubular apoptosis, cellular casts and tubular injury were included. The scoring was as follows: 0 (<10%), 1 (10-25%), 2 (25-50%), 3 (50-75%) and 4 (>75%). The percentage of renal injury was quantified and conducted in a blinded manner.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. TUNEL is the method of using the TdT enzyme to covalently attach a tagged form of dUTP to 3'ends of double- and single-stranded DNA breaks in cells. It is a reliable and useful method to detect DNA damage and cell death *in situ*. In the present study, renal samples were collected and fixed in 4% paraformaldehyde at room temperature for 24 h, paraffin embedded, and sectioned at 4 μ m. Then the slides were deparaffinized, rehydrated and the antigen was exposed. The death of tubular cells was detected with Cell Death Detection kit (Fluorescein; Roche Diagnostics, Indianapolis, IN, USA) in accordance with the manufacturer's protocol. Incubated with the tunnel reagent at 37°C for 2 h and mounted with antifade. Image Pro-Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used for positive cell counting.

Immunohistochemistry (IHC) assay. Renal samples were collected and fixed in 4% paraformaldehyde at room temperature for 24 h, paraffin embedded, and sectioned at 4 μ m. Then, the slides were deparaffinized, rehydrated in a descending alcohol series and the antigen was exposed by 20 min of incubation at 100°C in sodium citrate. Thereafter, the slides were incubated with 3% H₂O₂ at 37°C for 10 min to block the endogenous peroxidase activity. Sequentially, the slides were incubated with bovine serum albumin (cat. no. abs957; Absin Bioscience Inc., Shanghai, China) and the primary antibodies (cleaved Caspase 3, cat. no. 9664, 1:200, Cell Signaling Technology, Inc., Danvers, MA, USA; MPO, cat. no. abs120616, 1:200, Absin Bioscience Inc.) overnight at 4°C. After incubation with biotin-labeled secondary antibody (abs957, Absin Bioscience Inc.) at room temperature for

Table I. Primers for qPCR detection.

Gene	Sequence 5'-3'
β -actin	F: GTGACGTTGACATCCGTAAAGA R: GCCGGACTCATCGTACTCC
IL-1 β	F: CCGTGGACCTTCCAGGATGA R: GGGAACGTCACACACCAGCA
IL-6	F: AGTTGCCTTCTTGGGACTGA R: TCCACGATTTCCCAGAGAAC
TNF- α	F: CATCTTCTCAAATTCGAGTGACAA R: TGGGAGTAGACAAGGTACAACCC

IL, interleukin; TNF, tumor necrosis factor.

10 min, the color was developed with 3,3'-diaminobenzidine at room temperature for 30 sec. Then, the tissues were imaged by fluorescence microscopy (Olympus Corporation). The staining was evaluated in a blinded manner.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was isolated using TRIzol reagent (#15596-026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then reverse-transcribed into cDNA by using a Transcriptor First-Strand cDNA Synthesis kit (#04896866001; Roche) in accordance with the manufacturer's protocol. SYBR Green (#04887352001; Roche) was used to quantify the PCR-amplification products. qPCR was performed under the following conditions: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 5 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C. β -actin was used as the reference gene. The threshold cycle values of the samples were measured using the 2^{- $\Delta\Delta$ C_q} data analysis method (11). The results were presented as mean \pm standard deviation and SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) was used in the analysis. The primer pairs used in this study are listed in Table I.

ELISA. The blood was collected and centrifuged (3,500 x g) at 4°C for 15 min. Thereafter, the supernatant was harvested and stored at -20°C. The protein levels of TNF- α (cat. no. EK0527), IL-1 β (cat. no. EK0394) and IL-6 (cat. no. EK0411) were detected by ELISA kits (Boster Biological Technology, Wuhan, China) at 450 nm in accordance with the manufacturer's protocol.

Western blot analysis. Total protein (kidney) was isolated using radioimmunoprecipitation lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China) and the concentration was detected with bicinchoninic acid reagent (cat. no. #23225, Thermo Fisher Scientific, inc.). Equal amounts of proteins (30 μ g) were loaded, proteins were separated with 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Thereafter, such proteins were blocked in 5% skim milk for 1 h and incubated overnight at 4°C with primary antibodies (Bax, Bcl-2, cleaved caspase-3, p-IkBa, IkBa, p-P65, P65 and GAPDH). Following incubation in secondary antibodies (cat.

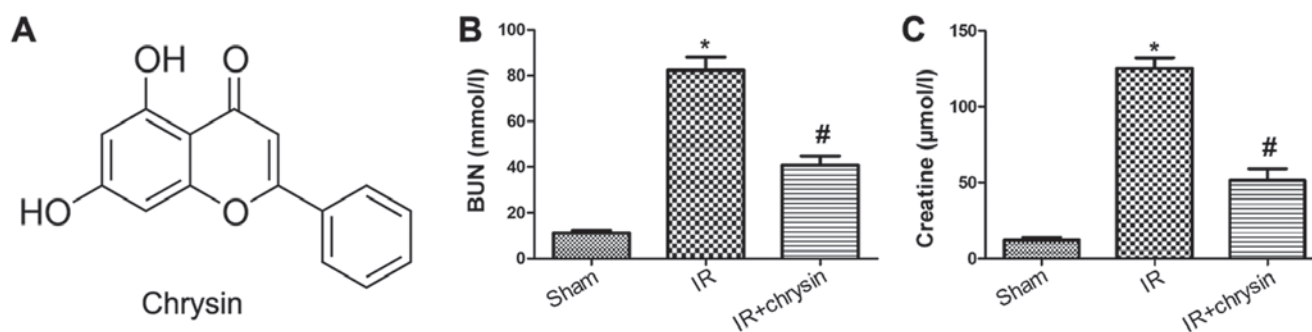


Figure 1. Chrysin pretreatment attenuates IR-induced renal dysfunctions. (A) The chemical structure of chrysin. The serum levels of (B) BUN and (C) creatinine in the sham, IR, and IR+chrysin group. * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the IR group. IR, ischemia reperfusion; BUN, blood urea nitrogen.

no. 926-32211; IRDye 800CW goat anti-rabbit IgG (H+L); LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature, washed three times and the membranes were scanned with the Odyssey infrared imaging system (LI-COR Biosciences).

Antibodies. The antibodies against Bax (cat. no. ab32503), Bcl-2 (cat. no. ab32124) and GAPDH (cat. no. ab181602) were obtained from Abcam and used in western blotting. The antibodies against cleaved caspase-3 (cat. no. 9661; used in IHC and western blotting) and phosphorylated (p)-I κ B α (cat. no. #2859; used in western blotting) were purchased from Cell Signaling Technology, Inc. The antibodies against I κ B α (cat. no. sc-371), P65 (cat. no. sc-372) and p-P65 (cat. no. sc-33020) were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA) and were used in western blotting. The antibody against myeloperoxidase (MPO; cat. no. abs120616; used in IHC) was purchased from Absin Bioscience Inc. The second antibody (cat. no. 926-32211; used in western blotting) was purchased from LI-COR Biosciences.

Statistical analysis. All experiments were repeated at least three times independently. Continuous data were expressed as mean \pm standard deviation. Difference between groups were statistically analyzed using one-way analysis of variance followed by Student-Newman-Keuls' method with SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Chrysin pretreatment attenuates IR-induced renal dysfunction. To investigate the effect of chrysin on renal functions in renal IR injury, the chrysin pretreatment mice were injected with chrysin (Fig. 1A) for 3 days (100 mg/kg each time) prior to the renal IR operation. The serum levels of BUN and Cr were significantly increased in the IR group compared with the sham group ($P < 0.05$). However, chrysin pretreatment significantly decreased the levels of BUN and Cr ($P < 0.05$; Fig. 1B and C). These results indicate that Chrysin pretreatment attenuates IR-induced renal dysfunction.

Chrysin pretreatment attenuates renal IR-induced morphological abnormalities. Following renal IR, more severe tubular damage was observed in the IR group compared with the sham

group. However, the abnormalities and tubular injury were significantly attenuated in the chrysin pretreatment group compared with in the IR group ($P < 0.05$; Fig. 2A and B). These results indicate that Chrysin pretreatment attenuates renal IR-induced morphological abnormalities.

Chrysin pretreatment attenuates renal IR-induced apoptosis of tubular cells. The results presented above demonstrate that chrysin administration attenuates renal tubular damage. To investigate the effect of chrysin on tubular cell apoptosis, TUNEL staining was performed. The number of TUNEL positive cells was significantly increased in the IR group compared with in the sham group ($P < 0.05$). However, chrysin pretreatment significantly decreased the number of apoptosis cells compared with the IR group ($P < 0.05$; Fig. 3A and B). Considering that cleaved caspase-3 protein is a marker of cell apoptosis and the protein level of cleaved caspase-3 was investigated by immunohistochemistry (IHC). Results demonstrated that the protein level of cleaved caspase-3 was significantly increased following renal IR injury ($P < 0.05$). However, the expression of cleaved caspase-3 was significantly decreased in the IR+chrysin group compared with the IR group ($P < 0.05$; Fig. 3C and D). These results indicate that Chrysin pretreatment attenuates renal IR-induced apoptosis of tubular cells.

Chrysin pretreatment decreases the expression of Bax and cleaved caspase-3 and increases the expression of Bcl-2 in renal IR injury. To detect the effect of chrysin on pro-apoptosis and anti-apoptosis associated proteins, a western blot assay was conducted (Fig. 4A). The protein levels of Bax and cleaved caspase-3 were increased and the expression of Bcl-2 was decreased in the IR group compared with the sham group. However, the protein levels of Bax and cleaved caspase-3 were significantly decreased, and the protein level of Bcl-2 was significantly increased in the chrysin pretreatment group compared in the IR group ($P < 0.05$; Fig. 4B-D). These results indicate that Chrysin pretreatment decreases the expression of Bax and cleaved caspase-3 and increases the expression of Bcl-2 in renal IR injury.

Chrysin pretreatment attenuates renal IR-induced inflammatory response. To investigate the effect of chrysin on renal IR induced inflammatory response, the level of myeloperoxidase (MPO) by IHC was detected. The expression of MPO was significantly increased in the IR group compared with the sham

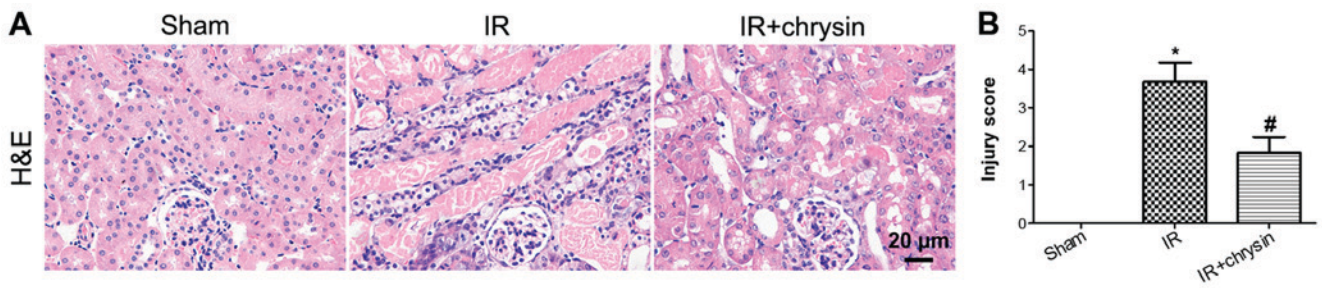


Figure 2. Chrysin pretreatment attenuates renal IR-induced morphological abnormalities. (A) The H&E staining of sham, IR and IR+chrysin group. Scale bar, 20 μm. (B) Tubular injury score of the sham, IR and IR+chrysin group. For assessment of renal injury, tubular apoptosis, cellular casts and tubular injury were included. Score 0-4 represent the injury area <10%, 10-25%, 25-50%, 50-75% and >75%. *P<0.05 vs. the sham group; #P<0.05 vs. the IR group. H&E, hematoxylin and eosin; IR, ischemia reperfusion.

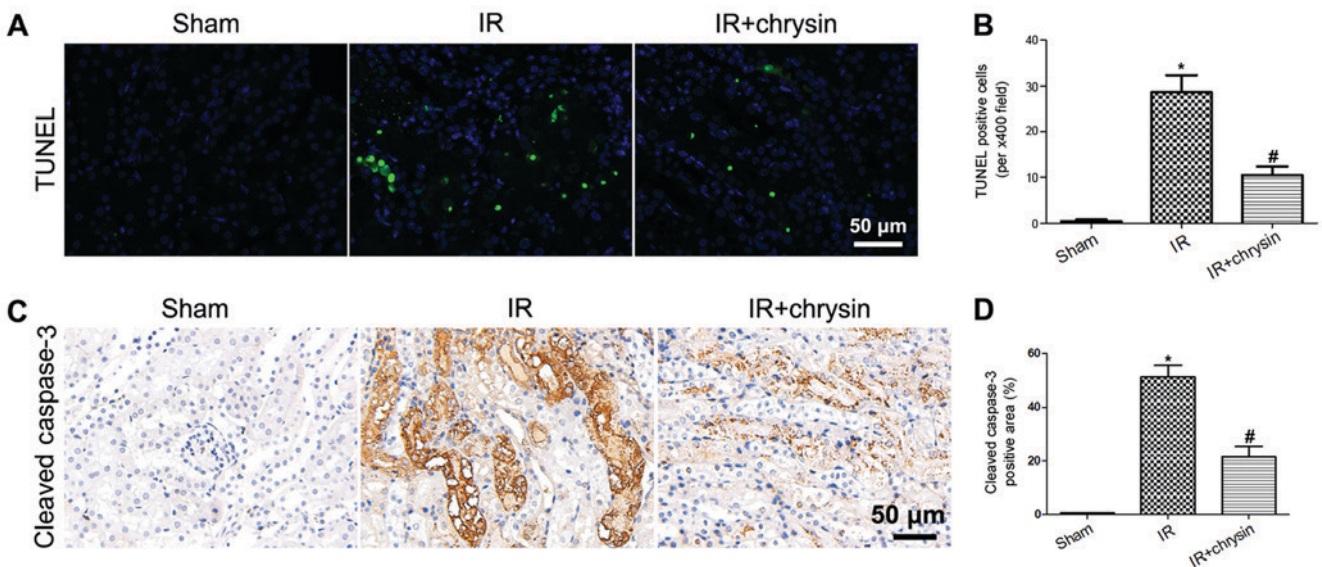


Figure 3. Chrysin pretreatment attenuates renal IR-induced apoptosis of tubular cells. (A) TUNEL staining of sham, IR and IR+chrysin group. Scale bar, 50 μm. (B) The number of TUNEL-positive cells of the sham, IR and IR+chrysin group were counted by Image-Pro Plus software version 6.0. (C) The immunohistochemistry staining of cleaved caspase-3 protein from the sham, IR and IR+chrysin group. Scale bar, 50 μm. (D) The positive areas of cleaved caspase-3 from the sham, IR and IR+chrysin group. *P<0.05 vs. the sham group; #P<0.05 vs. the IR group. IR, ischemia reperfusion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

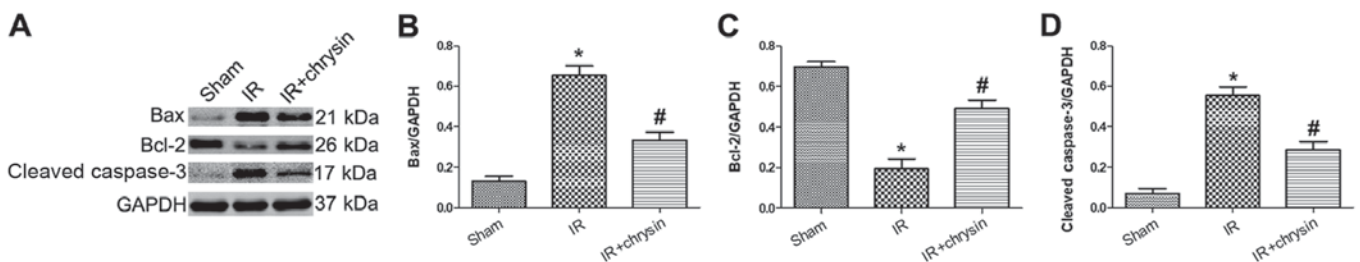


Figure 4. Chrysin pretreatment decreases the expression of Bax and cleaved caspase-3 and increases the expression of Bcl-2 in renal IR injury. (A) Western blotting of Bax, Bcl-2 and cleaved caspase-3 protein from sham, IR and IR+chrysin group. Relative protein levels of (B) Bax, (C) Bcl-2 and (D) cleaved caspase-3 from the sham, IR, and IR+chrysin group. *P<0.05 vs. the sham group; #P<0.05 vs. the IR group. IR, ischemia reperfusion.

group (P<0.05). However, the expression of MPO was significantly decreased in the chrysin pretreatment group compared with the IR group (P<0.05; Fig. 5A and B). Consistently, the mRNA levels of TNF-α, IL-1β and IL-6 were significantly decreased in the chrysin administration group compared with the IR group (P<0.05; Fig. 5C-E). Furthermore, the protein levels of proinflammatory cytokines were measured by ELISA.

Results demonstrated that the protein levels of TNF-α, IL-1β and IL-6 were significantly induced in renal ischemia reperfusion injury. However, chrysin pretreatment significantly decreased the expression of TNF-α, IL-1β and IL-6 in renal ischemia reperfusion injury (P<0.05; Fig. 5F-H). These results indicate that Chrysin pretreatment attenuates renal IR-induced inflammatory response.

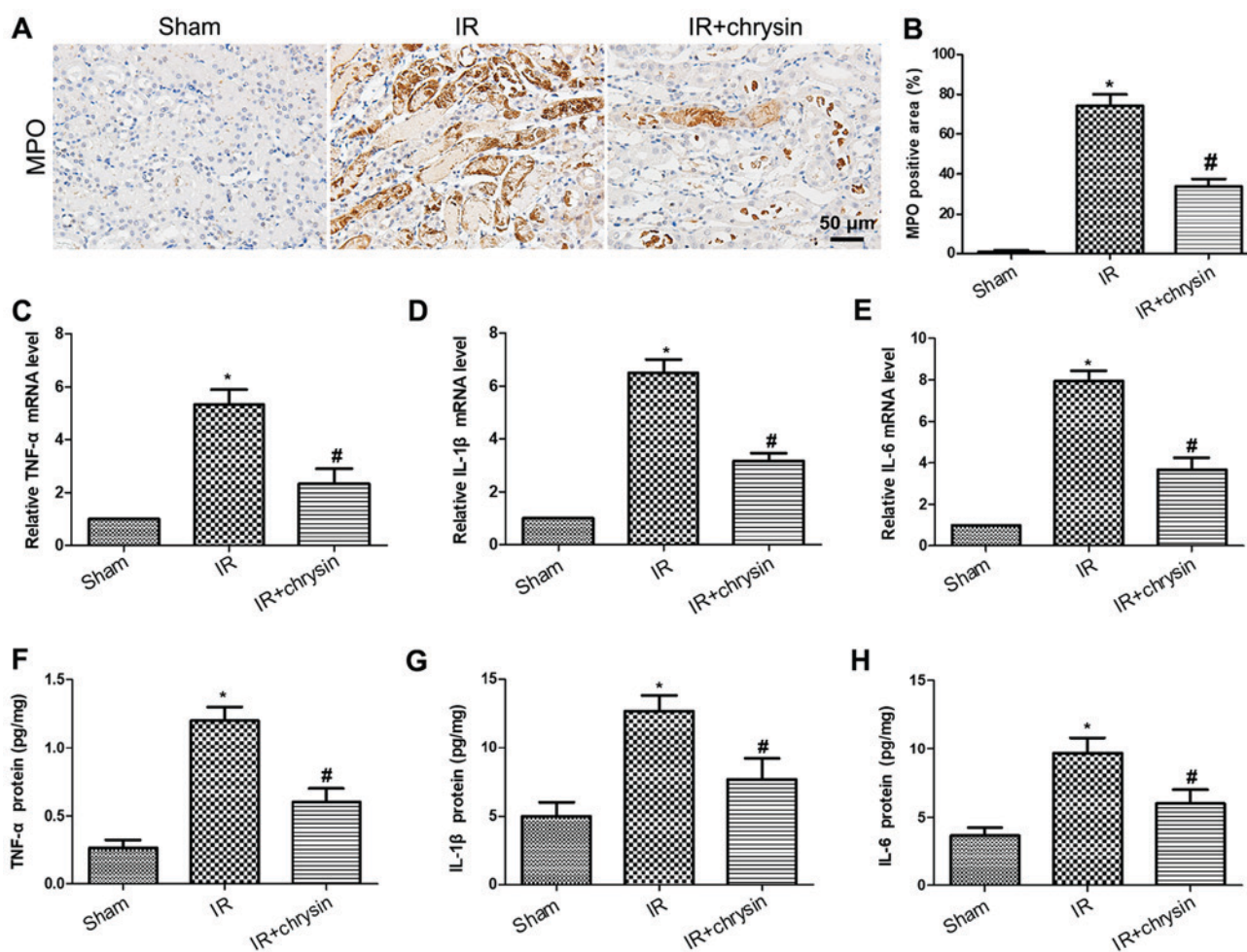


Figure 5. Chrysin pretreatment attenuates the renal IR-induced inflammatory response. (A) The immunohistochemistry staining of MPO in the sham, IR, and IR+chrysin group. Scale bar, 50 μ m. (B) The assessment of MPO positive areas in the sham, IR and IR+chrysin group. The mRNA levels of (C) TNF- α , (D) IL-1 β , and (E) IL-6 from the sham, IR, and IR+chrysin group by reverse transcription-quantitative polymerase chain reaction analysis. The serum protein levels of (F) TNF- α , (G) IL-1 β and (H) IL-6 from the sham, IR, and IR+chrysin group by ELISA analysis. *P<0.05 vs. the sham group; #P<0.05 vs. the IR group. IR, ischemia reperfusion; IL, interleukin; TNF, tumor necrosis factor; MPO, myeloperoxidase.

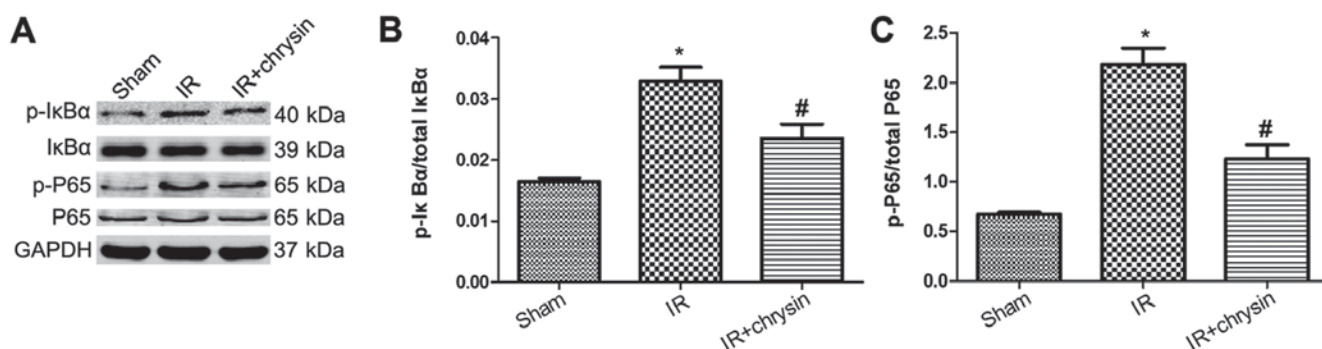


Figure 6. Chrysin pretreatment suppresses the NF- κ B signaling in renal IR injury. (A) Western blotting of I κ B α /P65 signaling pathway proteins in the sham, IR and IR+chrysin group. Relative protein levels of (B) p-I κ B α /total I κ B α and (C) p-P65/total P65 from the sham, IR, and IR+chrysin group. *P<0.05 vs. the sham group; #P<0.05 vs. the IR group. IR, ischemia reperfusion; NF, nuclear factor; p, phosphorylated.

Chrysin pretreatment suppresses the NF- κ B signaling in renal IR injury. The NF- κ B signaling is closely associated with renal IR injury (12). To investigate whether chrysin is involved in renal IR-induced NF- κ B signaling activation, the protein levels of I κ B α /NF- κ B signaling were measured (Fig. 6A). The phosphorylation levels of I κ B α and P65 were significantly

increased in the IR group compared with the sham group (P<0.05). However, the phosphorylation levels of I κ B α and P65 were significantly decreased in the chrysin pretreatment group compared with the IR group (P<0.05; Fig. 6B and C). These results indicate that Chrysin pretreatment suppresses the NF- κ B signaling in renal IR injury.

Discussion

Ischemic AKI is a complex syndrome with multiple cellular abnormalities, resulting in accelerating cycles of tubular apoptosis, inflammation and renal injury (13). Clinically, no agent can effectively prevent renal injury following ischemia (14). In this study, the protective effects of chrysin on IR-induced renal dysfunctions and morphological abnormalities were reported. Furthermore, chrysin attenuated tubular cell apoptosis and inflammatory response in renal IR injury.

Chrysin is a bioflavonoid with anti-inflammatory (15,16), anti-oxidant (17) and anti-carcinogenic (18) properties. In this study, a renal IR model was established in mice and pretreated with chrysin. Chrysin attenuated IR-induced renal dysfunction and morphological abnormalities. Chrysin particularly suppressed tubular apoptosis, inflammation and the $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling pathway in renal IR injury.

Tubular cell apoptosis is a major pathogenic mechanism in ischemic AKI (19). The outer medulla suffers the most severe injury and apoptosis of tubular cells leading to renal dysfunction in renal IR injury (5). The levels of Bax and cleaved caspase-3 increase and the level of Bcl-2 decrease in renal IR injury, resulting in tubular cell apoptosis (20,21). In the present study, the protein levels of Bax and cleaved caspase-3 were decreased and the protein level of Bcl-2 was increased in the chrysin pretreatment group compared with the IR group. Consistent with these results, the number of TUNEL positive cells was decreased in the chrysin pretreatment group compared with the IR group. The results of the present study suggest that chrysin protects against renal tubular cell apoptosis induced by renal IR in mice.

The inflammatory response is another important part of the pathophysiology implicated in renal IR injury (22). Following renal IR, neutrophil infiltration markedly increases and pro-inflammatory cytokines, including $\text{TNF-}\alpha$, IL-6 and IL-1 β , are induced (23). In the present study, neutrophil infiltration and $\text{TNF-}\alpha$, IL-6, and IL-1 β levels were decreased in the chrysin pretreatment mice compared with the IR group mice. The $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling pathway serves an important role in the renal IR-induced inflammatory response (12). In the present study, the phosphorylation levels of $\text{I}\kappa\text{B}\alpha$ and P65 were increased in the IR group compared with the sham group. However, the phosphorylation levels of $\text{I}\kappa\text{B}\alpha$ and P65 were significantly decreased in the chrysin pretreatment group compared with the IR group. Results suggest that chrysin functions as an anti-inflammatory agent in renal IR injury by suppressing the $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling pathway.

In conclusion, to the best of our knowledge this study provides the first evidence that chrysin attenuates renal dysfunction and morphological abnormalities in ischemic AKI. Furthermore, chrysin suppresses tubular cell apoptosis, inflammatory response and the $\text{NF-}\kappa\text{B}$ signaling pathway in renal IR injury. Therefore, chrysin may be a promising therapeutic agent for renal IR injury.

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Availability of data and materials

The data and materials are available from the corresponding author on reasonable request.

Authors' contributions

DL designed the research. MX performed the experiments and wrote the manuscript. HS analyzed the data. All authors read and approved the manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee at Hubei University of Arts and Science. The surgical procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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