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ORIGINAL ARTICLE

The beneficial effects of dose-dependent myricetin application on renal ischemia and reperfusion injury in a rat model

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Abstract

Background This study was aimed to evaluate the renoprotective effects of Myricetin at two dosages (50 and 100 mg/kg) within an experimental I/R injury model. The analysis focused on key immunological and oxidative stress pathways, including the regulation of pro- and anti-inflammatory cytokines, NF-κB and TLR4 signaling, as well as biomarkers of oxidative stress such as nitrate (NO), malondialdehyde (MDA), glutathione (GSH), nuclear factor (erythroid-derived 2)-like 2 (NRF2) and Heme oxygenase-1 (HO-1).

Methods and results Thirty female Wistar albino rats were randomly assigned to six groups. Myricetin was administered via intragastric gavage for seven days before ischemia/reperfusion. Renal ischemia was induced by clamping the left renal pedicle for 45 min, followed by a 3-hour reperfusion period. Kidney tissues were collected for histopathological, immunohistochemical, and biochemical analyses. Serum cytokine levels were measured by ELISA, while protein expressions were evaluated by Western blotting. I/R injury significantly increased pro-inflammatory cytokines and oxidative stress markers, alongside heightened NF-κB immunopositivity and TNF-α, IL-1β expression. Myricetin administration reduced pro-inflammatory cytokines, increased anti-inflammatory cytokines, and enhanced antioxidant responses. Immunohistochemical and Western blot analyses showed decreased NF-κB positivity and increased NRF2 expression.

Conclusions The renoprotective effects of Myricetin appear to be mediated by inhibiting the NF-κB pathway, reducing inflammation, and enhancing antioxidant responses. Furthermore, Myricetin activates the NRF2/HO-1 pathway, suggesting its potential as a therapeutic agent for ischemia/reperfusion-induced kidney injury.

Keywords Renal ischemia/Reperfusion · Myricetin · Apoptosis · Oxidative stress · Cytokines

Introduction

Renal ischemia/reperfusion (I/R) injury represents a primary contributor to the development of acute kidney injury (AKI), frequently arising in clinical scenarios including kidney transplantation, major surgical procedures, cardiovascular operations, and septic conditions [1]. Renal I/R injury is a complex pathophysiological process that begins with reduced oxygen and nutrient supply during ischemia, followed by the activation of reactive oxygen species (ROS) and inflammatory responses upon reperfusion [2]. This condition leads to significant impairments in

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kidney function and may result in chronic kidney disease (CKD) in the long term [3].

Renal I/R injury is fundamentally a biphasic pathological process characterized by ischemia followed by reperfusion. During ischemia, inadequate blood flow to renal tissues severely impacts cellular metabolism. ATP depletion, ion imbalances, and cellular swelling contribute to necrosis and apoptosis in renal tubular cells [4]. The reperfusion phase, characterized by a sudden influx of oxygen, leads to a rapid increase in reactive oxygen species (ROS) production and activation of inflammatory cells, exacerbating tissue damage. Consequently, microvascular dysfunction, endothelial injury, and the release of proinflammatory cytokines result in kidney function loss [5, 6].

The mechanisms underlying renal I/R injury include oxidative stress, inflammation, endothelial dysfunction, and cell death. Excessive ROS production leads to DNA damage and mitochondrial dysfunction, accelerating cell death [6, 7]. Moreover, the activation of inflammatory responses is evident through increased levels of mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10), which further promote tissue damage and renal tubular dysfunction [8, 9].

Apoptotic cell death plays a crucial role in renal I/R injury. Apoptosis is a programmed cell death process triggered by both the mitochondrial (intrinsic) and death receptor-mediated (extrinsic) pathways. The release of cytochrome c and activation of caspases are key factors in the initiation of the mitochondrial apoptotic pathway. During the reperfusion phase, increased oxidative stress enhances apoptotic signaling, leading to widespread cell death [3]. The pathophysiology of renal I/R injury is complex and apoptosis plays an important role in this process. Apoptosis is an important feature of tubular epithelial cell damage after I/R injury [10, 11]. Bcl-2 is a proto-oncogene that can inhibit apoptosis and is known to inhibit apoptosis induced by etiological factors that trigger ischaemia and reperfusion injury [12, 13]. The Bcl-2 family includes members that inhibit apoptosis (such as Bcl-2) or promote apoptosis (such as Bax). Overexpression of Bcl-2 may protect against renal IRI [14, 15]. Caspase-3 is an important enzyme involved in inflammation and apoptosis and is also involved in renal IRI [16, 17]. Activation of caspase-3 is an important marker of apoptosis and is increased in IRI. Inhibition of caspase-3 may have protective effects against renal I/R injury [13, 16, 18].

Toll-like receptor 4 (TLR4), a key component of the innate immune system, plays a significant role in renal I/R injury. TLR4 is activated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [19]. The activation of DAMPs triggers the Nuclear Factor Kappa-B (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways, amplifying the inflammatory response and promoting apoptosis [1]. Studies have shown that TLR4 is activated during renal I/R, mediating inflammation and cellular death mechanisms [20]. Inhibition of the TLR4 signaling pathway has been demonstrated to reduce renal function loss and suppress inflammatory responses [5].

Among non-pharmacological approaches, ischemic preconditioning (IP) has gained attention. IP involves brief cycles of ischemia and reperfusion to enhance tissue resistance against prolonged ischemia. Additionally, stem cell therapies and cellular regenerative approaches hold promise as alternative strategies for restoring kidney function [3].

Flavonoids, a class of naturally occurring polyphenolic compounds, are ubiquitously found throughout the plant kingdom, including various fruits, vegetables, and roots [21]. Among them, Myricetin (MYR), a flavonoid prevalent in natural plant sources such as members of the *Lauraceae* family, including bayberry, has garnered significant research interest. Extensive pharmacological investigations have elucidated MYR's diverse biological properties, highlighting its potential for further scientific exploration and therapeutic applications [22]. Notably, MYR has been reported to exhibit a wide range of pharmacological activities, including anti-inflammatory, antitumor, antibacterial, antiviral, and anti-obesity effects. Furthermore, it has demonstrated protective roles in cardiovascular health, neuroprotection, and hepatoprotection [23–26]. Comparative analyses of flavanols have also suggested that MYR functions as a potent free radical scavenger, emphasizing its role in oxidative stress modulation [27].

This study was aimed to evaluate the renoprotective effects of MYR at two dosages (50 and 100 mg/kg) within an experimental I/R injury model. The analysis focused on key immunological and oxidative stress pathways, including the regulation of pro- and anti-inflammatory cytokines, NF-κB and TLR4 signaling, as well as biomarkers of oxidative stress such as nitrate (NO), malondialdehyde (MDA), glutathione (GSH), nuclear factor (erythroid-derived 2)-like 2 (NRF2) and Heme oxygenase-1 (HO-1). These parameters were assessed using IHC, ELISA, tissue biochemistry, and Western blotting. Renal I/R injury remains a critical factor contributing to AKI and kidney failure, posing substantial clinical challenges and being associated with high morbidity and mortality. Elucidating its underlying molecular mechanisms is essential for developing targeted therapeutic strategies. Future research should explore innovative therapeutic approaches aimed at modulating oxidative stress and inflammation, as well as emerging regenerative medicine techniques to enhance renal resilience against I/R-induced damage.toparlanayım.

Methods

Ethical statement and experimental procedures

Approval for this study was granted by the Local Animal Experimentation Ethics Committee at Kafkas University in Kars (Approval No: KAU-HADYEK 2024/118). All procedures involving animals were conducted in accordance with institutional and national guidelines, and complied with the European Directive 2010/63/EU for animal experiments.

The study was conducted using 30 female Wistar Albino rats, each weighing between 200 and 300 g and aged 12 weeks. The rats were Housed under controlled environmental conditions, which included a 12-hour light/dark cycle, an average temperature of 22 ± 2 °C, and a humidity level of 60 ± 5%. Throughout the experimental period, the rats were provided with unrestricted access to tap water and standard laboratory food. They were randomly assigned into six groups as seen in Table 1.

Myricetin (MedChemExpress, Cat. No.: HY-15097 Purity: 98.42%) was dissolved in sterile double-distilled water in two doses (50 mg/kg, 100 mg/kg) and administered to rats Simultaneously by intragastric gavage for 7 days [28]. Then, the rats were fasted 12 h prior the surgery. Prior to the surgical procedures, all rats were anesthetized with ketamine hydrochloride (15 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). After the rats were anesthetized, their abdomens were shaved with a razor and a midline incision of approxmately1-1.5 cm was made under sterile conditions [29, 30]. Then the rat's abdominal region was sterilized with povidone-iodine solution. Following a midline incision, the left kidney was palpated and its renal pedicle was isolated. The left kidney was palpated and the left renal pedicle was isolated after a midline incision. The right kidney was removed and the membrane in the kidney capsule was peeled off. The right renal artery and vein were ligated with 3.0 Silk thread for 45 min to create ischemia, followed by a three-hour reperfusion period. Successful occlusion was confirmed by a marked pallor of the kidney, which reverted to its normal reddish color following reperfusion. Postoperatively, povidone-iodine solution was applied locally to the midline incision of the abdominal cavity [31]. At the end of the reperfusion period, animals were euthanized by intraperitoneal injection of high doses of anesthesia (60 mg/kg ketamine hydrochloride, 40 mg/kg xylazine). Subsequently, 5 ml of blood was collected into the tubes, and kidney tissues were rapidly excised. Half of the kidney tissues were stored − 80 °C for tissue biochemistry analysis; the other half were fixed in 10% formalin solution for histopathological analyses [32].

Table 1 All experimental groups

No	Groups	*N*	Abbreviations
1	Control	6	Cnt
2	Sham	6	Sham
3	I/R	6	I/R
4	Myricetin 50 mg/kg before I/R	6	I/R + M50
5	Myricetin 100 mg/kg before I/R	6	I/R + M100

Histopathological and immunohistochemical analysis

Following excision, each kidney was bisected, and one half was fixed in 10% neutral buffered formalin for 72 h. Following this, the tissue was subjected to dehydration through a graded series of alcohol concentrations (70%, 80%, and 100%) and subsequently embedded in paraffin. Kidney tissues were sectioned into 5-µm slices using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). The sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin (H&E). Histological evaluation of the kidney sections was conducted by examining approximately 10 randomly selected fields under a 40x objective lens. The analysis was performed by a pathologist blinded to the treatment groups [33].

In this study, IHC staining was performed to identify the presence of NF-κB p65 protein. The staining procedure followed a series of sequential steps to ensure accurate and consistent results. Sections mounted on Poly-L-Lysine-coated slides were incubated in an oven at 60 °C overnight to secure the tissue to the slides and to facilitate the removal of paraffin. After tissue transparanting procedures, they were sequentially rehydrated by passing through a graded alcohol series. Finally, the sections were rinsed in distilled water to remove residual alcohol and rehydrate the tissues. In order to re-expose the antigen surfaces blocked in the tissues as a result of fixation with formaldehyde, they were kept in sodium citrate solution under high pressure and temperature for 1–2 min. The tissues were kept in 3% H_2O_2 for 10 min to inactivate endogenous peroxidases in the tissue. In order to prevent antibody binding to other proteins, protein block solution was dripped into the tissues and kept for 5 min. Then, the excess of the block solution was poured and monoclonal NF-κB (Catalogue number: sc-8008, Santa Cruz Biotechnology Inc., Texas, USA) primary antibody diluted 1:100 was dropped into the tissues to be kept at 4 °C for 1 night. The following day, the slides were first washed with PBS and the sections were then incubated with a biotinylated secondary antibody for 10 min at room temperature. Subsequently, the slides were incubated with streptavidin-peroxidase solution for 10 min. 3,3'-Diaminobenzidine (DAB) chromogen was applied to the tissue sections. Then, the sections were counterstained with Mayer's hematoxylin for 1 min to provide background staining. Finally, the slides were mounted with coverslips using Entellan. The prepared

slides were examined under a light microscope (Olympus CX41) [34, 35].

Biochemical analysis

For the analysis of GSH levels, precisely 200 µL of kidney tissue homogenate was carefully transferred into a designated test tube. Simultaneously, an equal volume (200 µL) of the prepared standard solution was placed into a separate tube, specifically allocated for the standard reference. Following this step, 1.8 mL of distilled water and 3 mL of the precipitating solution were sequentially added to each corresponding tube to facilitate the reaction process. In parallel, for the blank sample preparation, a mixture of 800 µL of distilled water and 1.2 mL of the precipitating solution was prepared in a separate tube. Once all components were combined, the tubes were gently but thoroughly mixed to ensure uniformity and then incubated in an ice-water bath for a duration of 5 min to stabilize the reaction conditions. Following the incubation period, centrifugation was carried out at a speed of 3000 rpm for 10 min to separate the precipitate from the supernatant. After centrifugation, precisely 2 mL of the resulting supernatant was carefully collected from each tube, including those containing the standard, test, and blank samples. To each of these supernatant fractions, 8 mL of phosphate-buffered saline (PBS) was added, and the solutions were homogenized to achieve a consistent reaction environment. Finally, a volume of 1 mL of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) was introduced into each sample to facilitate the colorimetric reaction. The optical density (OD) of both the standard and test samples was subsequently measured at a wavelength of 412 nm, using the blank solution as the reference for baseline correction [36].

For the quantification of MDA, separate test and blank tubes were prepared. Each test tube received 0.5 ml of tissue homogenate. Subsequently, 2.5 ml of 20% trichloroacetic acid (TCAA) was added to the test tubes, while the blank tubes received 3 ml of the same reagent. Following this, 1 ml of thiobarbituric acid (TBA) solution was added to both sets of tubes. The mixtures were then incubated in a water bath at 90 °C for 30 min. After incubation, the tubes were allowed to cool to room temperature. Next, 4 ml of n-butanol was added to each tube, followed by centrifugation at 3000 rpm for 10 min. The upper n-butanol layer was carefully transferred into new tubes, and absorbance was measured at 535 nm using a spectrophotometer, with the blank serving as a reference. MDA concentrations were calculated using a standard calibration curve [37].

For NO analysis, 100 µL of sample was pipetted into the microplate wells. 100 µL of VaCl3 was added to all wells. Immediately afterwards, 100 µL of Griess reagent was pipetted. After incubating for 30 min at 37 °C in an oven, absorbances were read at 540 nm wavelength. For nitrite analysis, 100 µL of sample was pipetted into the microplate wells. Immediately afterwards, 100 µL of Griess reagent was pipetted. After incubating for 30 min at 37 °C in an oven, absorbances were read at 540 nm wavelength. The nitric oxide (NO) concentration was found by adding the nitrate and nitrite concentrations found by looking at the calibration curve [38].

ELISA analysis

In this study, 5 mL blood samples were collected from the apex of the heart ventricle of rats and transferred to sterile blood collection tubes to determine the levels of cytokine parameters. The blood samples were then centrifuged at 3000 rpm for 15 min at +4 °C in a refrigerated centrifuge device until a clear supernatant was obtained. The serum supernatant samples were aliquoted into sterile eppendorf tubes and stored at −80 °C until the experiment day. We aimed to detect the levels of six different cytokines (4/6 was pro-inflammatory and 2/6 was anti-inflammatory). For this reason, we used ELISA technique and the following ELISA kits according to the manufacturers' recommendation: Rat TNF-α (Cat. No: E0764Ra BT Lab, Shanghai, China), Rat IL-1ß (Cat. No: E0119Ra BT Lab, Shanghai, China), Rat IL-6 (Cat. No: E0135Ra BT Lab, Shanghai, China), Rat IL-10 (Cat. No: E0108Ra BT Lab, Shanghai, China), Rat IL-15 (Cat. No: E0113Ra, BT Lab, Shanghai, China), IL-18 (Cat. No: E0117Ra, BT Lab, Shanghai, China) and Rat TGF-ß (Cat. No: E0778Ra, BT Lab, Shanghai, China). The results were read at 450 nm absorbance in the ELISA Reader (Thermo MultiSkan GO) and OD values were noted [26].

Western blot analysis

The kidney tissue samples were weighed and then grounded using liquid nitrogen. The homogenization process was carried out using a tissue lyser (TissueLyser LT, Qiagen, Germantown, MD, USA) at 30 Hz for 20 s. Total protein levels (Pierce BCA protein assay kit, ThermoFisher Scientific, Waltham, MA, USA) were measured in tissue samples before WB, at least 40 µg/well sample was used. 40 µg of protein samples were electrophoresed using 8–12% SDS-PAGE, and the separated proteins were transferred to PVDF membrane, primary antibody which were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) were used for overnight at 4 °C NF-κB, Bcl-2, Caspase-3, NRF2, HO-1, TLR4 and β-actin at a concentration of 5 µg/mL prepared in blocking buffer was added to the membrane and incubated overnight in the refrigerator on an orbital mixer. Secondary

Table 2 Primary antibodies used in Western blot analysis

Antibody	Manufacturer	Dilution
Caspase-3	Santa Cruz, sc- 56,053	1/1000
Bcl-2	Affinity Biotech, AF4650	1/1000
TLR4	Santa Cruz, sc-293,072	1/1000
NF-κB-p65	Santa Cruz, sc-109	1/1000
HO-1	Santa Cruz Sc-10,789	1/1000
NRF2	Santa Cruz Sc-365,949	1/1000
Beta-actin	Santa Cruz, sc-47,778	1/2000
Secondary antibody (Goat anti-rabbit IgG-HRP)	Santa Cruz, sc-2004	1/2000
Secondary antibody (Mouse anti-rabbit IgG-HRP)	Santa Cruz, sc-2357	1/2000

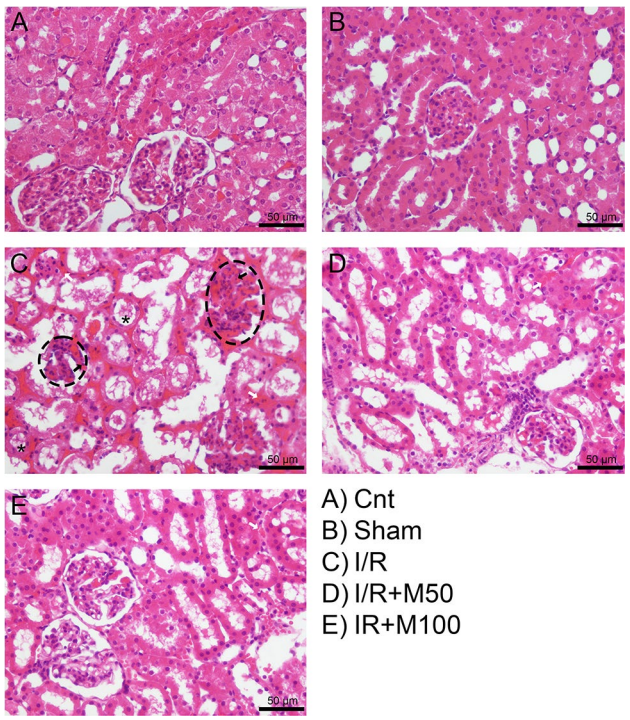


Fig. 1 Histological examinations of kidney tissue all experimental groups. The circle area: Necrotic glomeruli, Black Arrow: Picnotic, Asterisk: Diluted tubule and, White Arrow: İntertubular Hemorrhage. (**A**) Control group, (**B**) Sham, (**C**) I/R, (**D**) I/R + M50, (**E**) I/R + M100, *Dye*: Hematoxylin & eosin; *Bar*: 50 µm)

antibody (sc-2004/sc-2005) at a concentration of 1 µg/mL prepared in blocking buffer was then added and incubated for 1 h at room temperature. Catalog numbers and dilution rates of the kits are presented in Table 2. Finally, chemiluminescent HRP substrate prepared according to the manufacturer's recommendations was added to the membrane and the blots were photographed in chemiluminescent mode with a gel imaging system (Bio-Rad). The total amount of protein obtained from gel analysis of a sample was divided by the amount of the blot on the membrane and normalization was performed (Total protein normalization) [26].

Results

Histopathological findings on kidney tissue

The proximal and distal tubules and kidney glomeruli, which are components of the cortex, were examined in detail for histopathological changes. Accordingly, the kidney glomeruli had regular appearance in control group. The Bowman space was within physiological dimensions. The cells forming the glomeruli had a healthy appearance. The cells forming both the proximal and distal tubules were regular in shape and formed regular tubules with borders (Fig. 1A). In sham group kidney micrographs, unlike the control, very little eosinophilia was noted in the cytoplasm of the cells, while the tubular structures and glomeruli were normal in appearance (Fig. 1B). The most dramatic damages were seen in I/R group. In the necrotic glomeruli, the nuclei of the cells were pyknotic and their cytoplasm was intensely eosinophilic. The lumens of proximal and distal tubules appeared dilated, with thinning of the tubular walls. In addition, hemorrhage was observed in the intratubular spaces (Fig. 1C). It was determined that hemorrhage was significantly reduced in the Myricetin-treated groups and the glomeruli were more regular compared to the ischemia group (Fig. 1D–E).

Immunohistochemical examinations on kidney sections

Kidney sections were stained with a primary NF-κB antibody for IHC analysis. Immunonegative staining can be seen in control group (Fig. 2A). A few immunopositive cells were observed at tubules cell in control and sham groups (Fig. 2B–C). In I/R group, a large number of positive cells were detected, especially in tubules and glomeruli (Fig. 2D). It was determined that the number of immune-positive cells in M50 group was in a range close to I/R group (Fig. 2E), but in M100 group, the immunopositive density was reduced compared to I/R group (Fig. 2F). On the other hand, semi-quantitative scoring of histopathological analyses is available in Table 3.

Biochemical findings

According to the results, the highest and the lowest renal NO and MDA levels were detected in I/R and Control groups, respectively (Fig. 3). On the other hand, the highest and the lowest renal GSH levels were detected in Control and I/R groups, respectively (Fig. 3). Significant differences were observed between the Control group and the experimental groups, as well as between the I/R group and the Myricetin-treated groups. All statistical analysis results are shown in

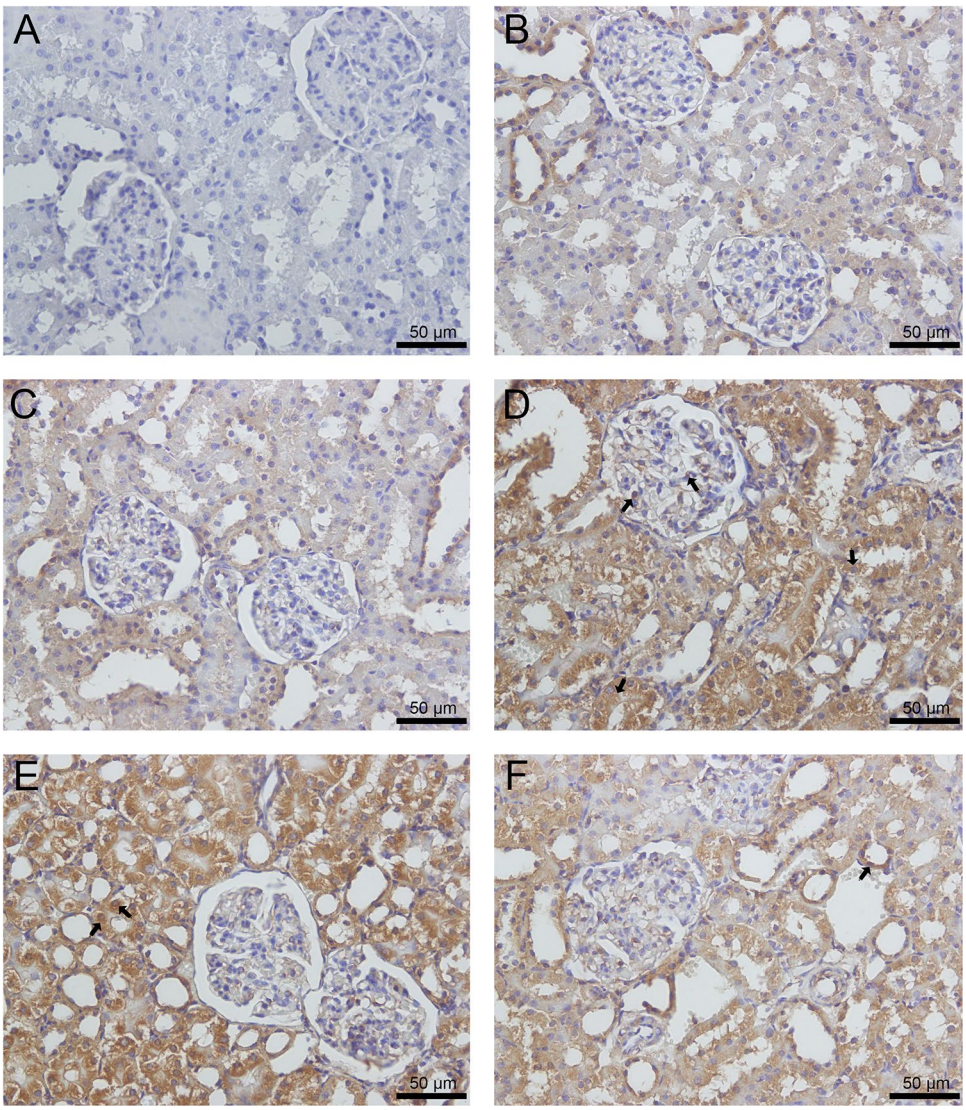


Fig. 2 NF-κB positivity in kidney tissues of all experimental groups. *Black Arrow*: Immune positive cell. (**A**) Control (−), (**B**) Control (+), (**C**) Sham, (**D**) I/R, (**E**) I/R + M50 (**F**) I/R + M100, *Bar*: 50 µm)

Table 3 Semi-quantitative scoring of histopathological analyses

Groups	*n*	Glomerular degeneration	Tubular dilatation	Intertubular erythrocytes infiltration
Cnt	6	0	−	0
Sham	6	0	−	0
I/R	6	4	+++	4
I/R + M50	6	2	++	2
I/R + M100	6	1	++	2

0, none; 1, mild; 2, moderate; 3, severe; 4, more severe. Grade 0: −, Grade 1: + (0–33% mild), Grade 2: ++ (33–66% moderate), Grade 3: +++ (66–100% severe)

Fig. 3. In Fig. 3; * presents $p < 0.05$; ** presents $p < 0.01$; *** presents $p < 0.001$; and **** presents $p < 0.0001$;

ELISA findings

To evaluate the inflammatory response following renal I/R injury and the potential protective role of Myricetin, pro-inflammatory cytokine levels, including TNF-α, IL-1β, IL-6, IL-15, and IL-18, were measured in serum samples. The I/R group exhibited a significant increase in all measured pro-inflammatory cytokines compared to control group ($p < 0.001$). Also, Myricetin treatment at both doses (50 mg/kg and 100 mg/kg) attenuated this increase in a dose-dependent manner.

Notably, the I/R-M100 group showed the most substantial reduction in pro-inflammatory cytokines, particularly TNF-α and IL-1β levels, which were significantly lower compared to the untreated I/R group ($p < 0.0001$,

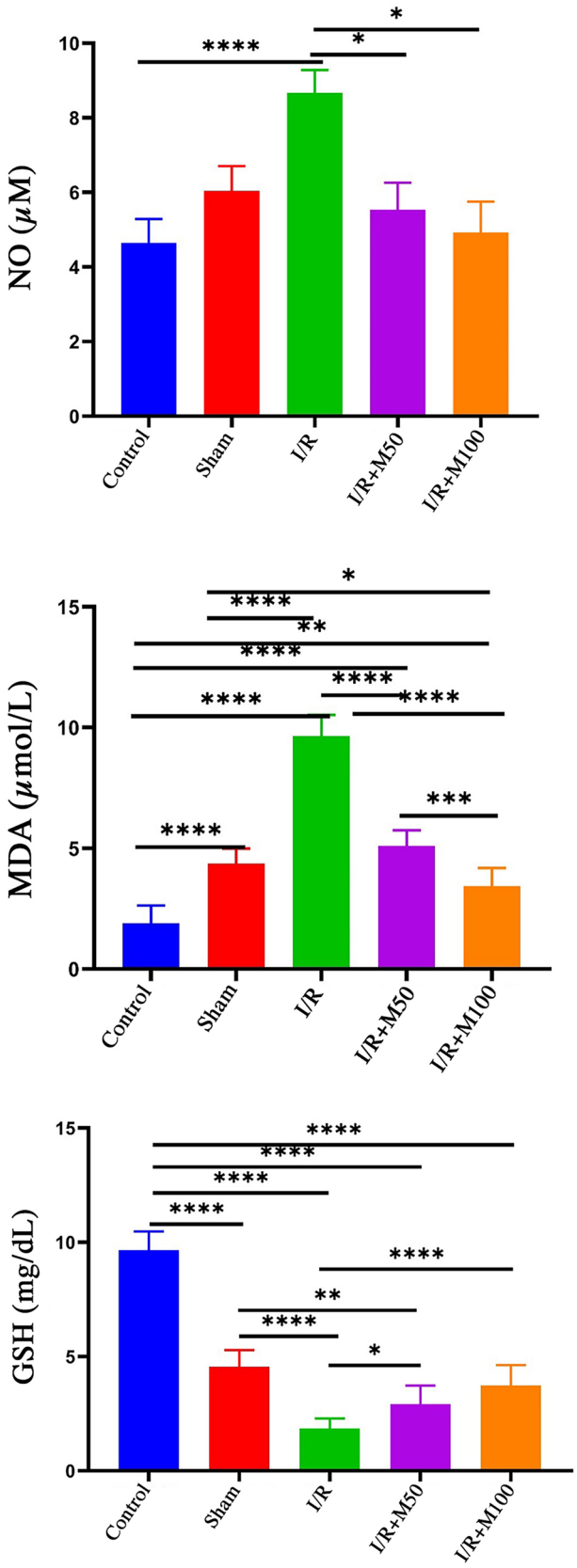


Fig. 3 Oxidative stress parameters of all experimental groups

Fig. 4A–B). Similarly, IL-6 and IL-15 levels were markedly decreased in Myricetin-treated groups, with the I/R-M100 group showing the most significant suppression ($p<0.001$, Fig. 4C–D). Although IL-18 levels increased significantly in the I/R group, Myricetin treatment failed to reduce IL-18 levels to control values, indicating a limited effect on this cytokine (Fig. 4E).

To assess the anti-inflammatory response, IL-10 and TGF-β cytokine levels were also analyzed. While IL-10 levels remained unchanged across all experimental groups, Myricetin administration resulted in a dose-dependent increase in TGF-β levels, with the highest elevation observed in the I/R-M100 group ($p<0.0001$, Fig. 4F). This suggests that Myricetin may facilitate tissue repair and immune regulation through the upregulation of TGF-β, a key mediator of anti-inflammatory and fibrotic processes.

Western blot findings

In the evaluation of kidney tissue protein expressions, Bcl-2 protein expression levels were highest in control group, significantly decreased in I/R group and partially increased in I/R + M100 ($p<0.05$). Caspase-3 protein expression reached the highest level in I/R group ($p<0.01$) and showed a significant decrease in I/R + M100 group. Additionally, NF-κB expression increased in I/R group ($p<0.01$); however, did not change in I/R + M100 group ($p>0.05$). Expression of HO-1 and NRF2 proteins reached the highest level in I/R group and showed a significant decrease in I/R + M100 group ($p<0.01$). TLR4 expression was also found at the highest level in I/R group ($p<0.0001$) and did not show a significant change with I/R + M100 treatment ($p>0.05$). Protein band intensities and comparisons of all groups are presented in Fig. 5.

Discussion

Renal I/R injury is one of the main causes of acute renal failure and is commonly seen in clinical situations such as surgical interventions and kidney transplantation. Renal I/R injury is characterized by decreased oxygen and nutrient supply during ischemia, increased ROS production during reperfusion and triggering of inflammatory response. This process may lead to loss of renal function and may develop into chronic kidney disease in the long term [39, 40].

This study was aimed to investigate the molecular mechanisms through which MYR influences inflammatory responses, oxidative stress, and cell death in the context of renal I/R injury. The results demonstrated that MYR effectively downregulates pro-inflammatory cytokines, enhances antioxidant defense systems, and modulates pathways

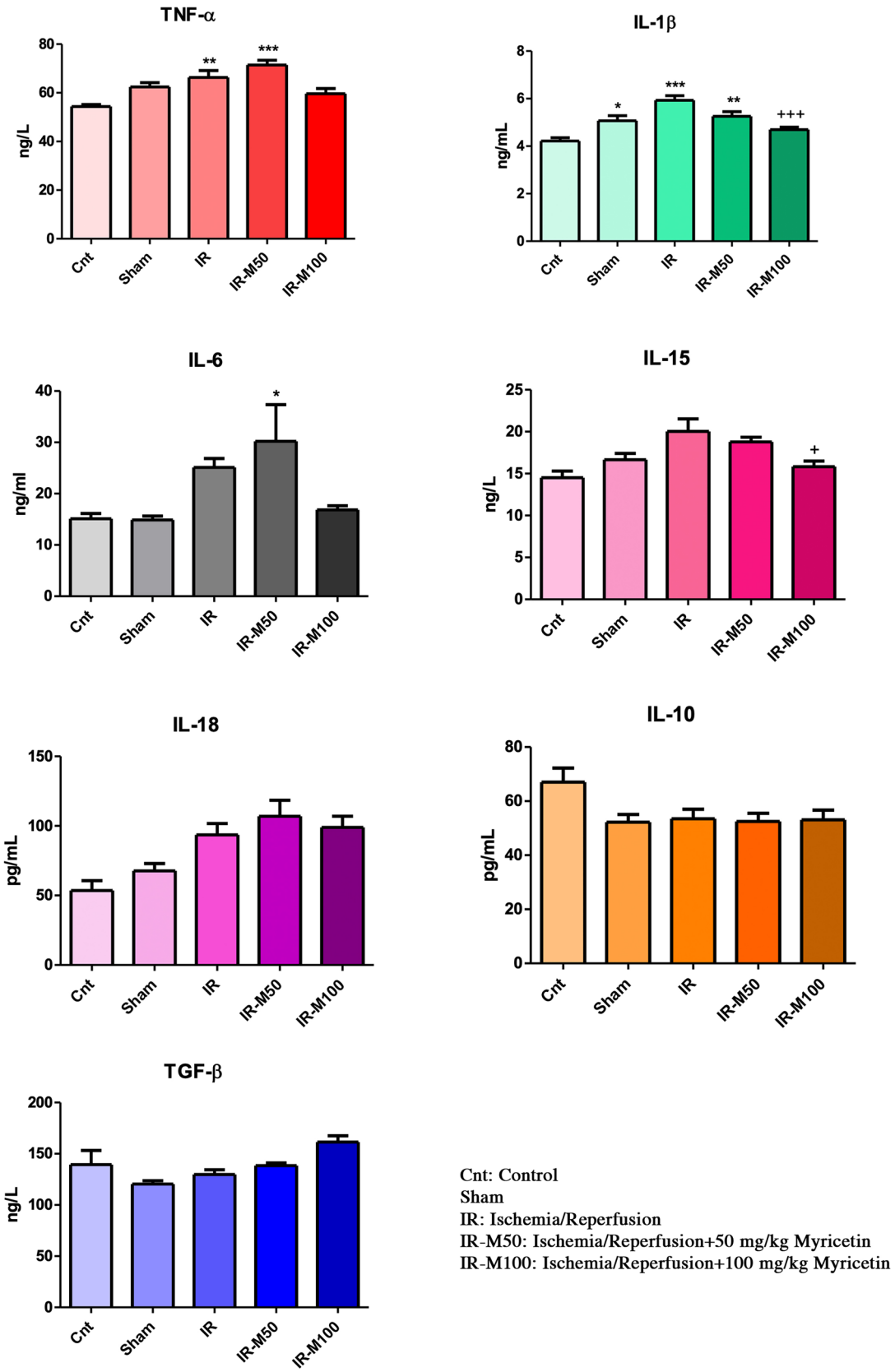


Fig. 4 The pro- and anti-inflammatory cytokine levels of all experimental groups

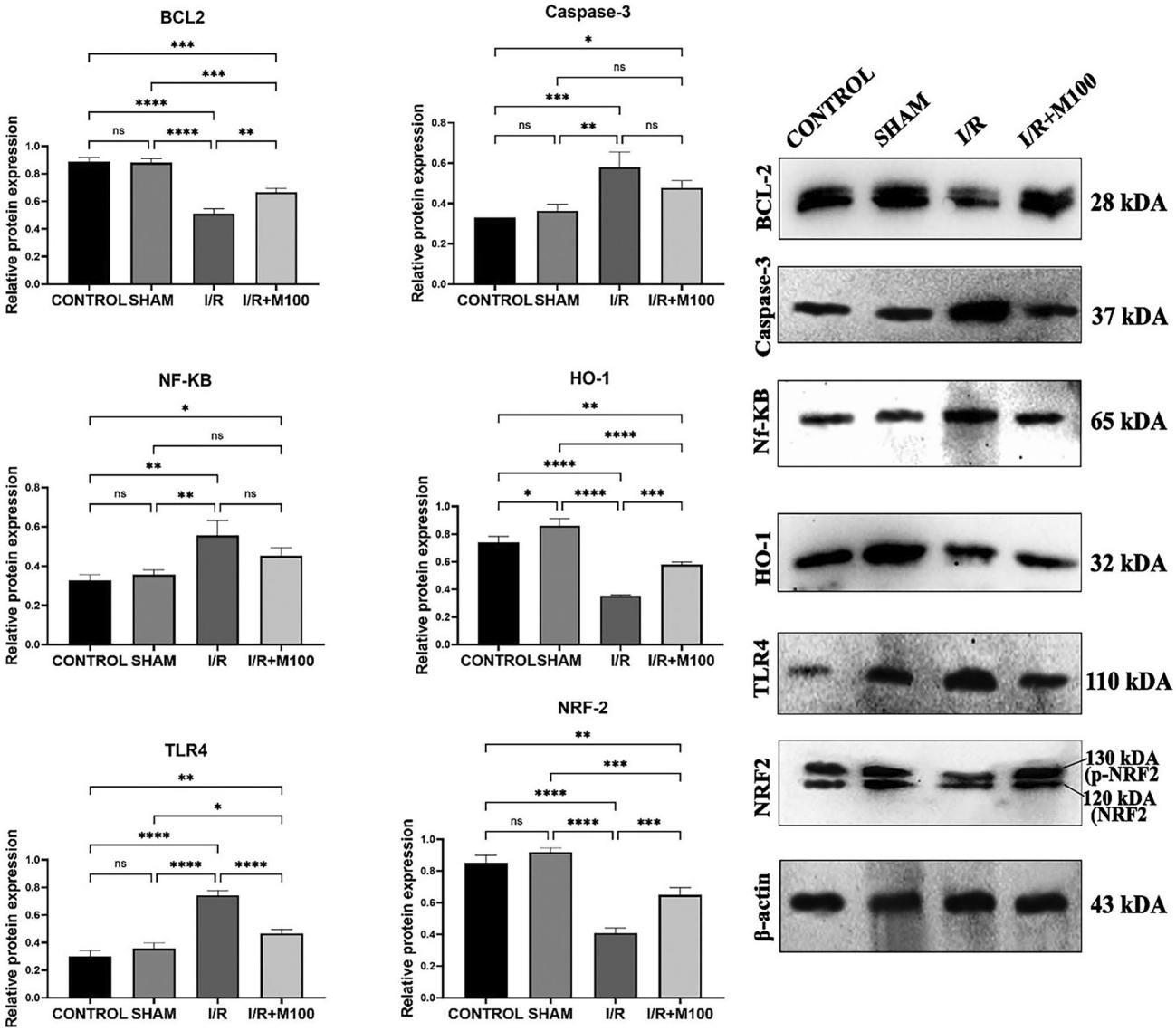


Fig. 5 The relative protein expression levels in kidney tissues across all groups. Quantitative analysis of Caspase-3, Bcl-2, NF-kB, HO-1, NRF-2, TLR4 and Beta Actin protein expression levels and Western blot results. Graphs show the relative protein expression levels of each group. Protein expression values of all groups are given as mean ± standard deviation. Statistical analyses were performed by One-Way ANOVA and Tukey post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.0001$, ****$p<0.00001$

involved in cellular death, indicating its potential renoprotective effects. MYR treatment caused decreases in the levels of pro-inflammatory cytokine and oxidative stress parameters while it was increased the levels of anti-inflammatory cytokine and anti-oxidant levels.

Histopathological examinations showed that renal I/R injury caused disruptions in glomerular structure, degeneration of tubular cells and intratubular hemorrhages. In MYR-treated groups, glomerular structures were found to be more regular and inflammatory cell infiltration decreased [41, 42]. IHC analyses revealed that NF-κB expression was significantly increased in I/R group; however, was suppressed by MYR treatment. NF-κB is known to exacerbate cellular stress responses by increasing inflammatory gene expression [43]. Wang et al. showed immunohistochemically that NF-κB expression increased in I/R and decreased in Picroside II-treated rats [44]. While MYR has inhibitory effects on the NF-κB pathway, additional sources detail the mechanisms of this inhibition and the effects of other agents (Picroside II, RP105, DHM, Total Flavonoids) on NF-κB. The findings obtained by immunohistochemistry support that NF-κB expression is increased in I/R injury and that agents such as Myricetin may provide protective effects by reducing this increase. The present findings support that MYR suppresses the pro-inflammatory response by suppressing NF-κB activation.

The current study demonstrated that oxidative stress plays a critical role in renal I/R injury. MDA and NO levels were significantly increased in I/R group, while GSH levels were decreased. MYR treatment decreased MDA and NO levels and increased GSH levels. These findings support that Myricetin reduces oxidative damage by activating the NRF2/Keap1/HO-1 signaling pathway as shown in previous studies [45, 46]. Total flavonoids have been shown to have a protective effect against I/R injury and activate the NRF2/HO-1 signaling pathway. Total flavonoids have been reported to affect the NRF2/HO-1 signaling pathway by increasing Sirt1 levels [47]. Apocynin has been reported to reduce renal tissue MDA and MPO levels in renal I/R injury [48]. Other studies have been reported that renal I/R-induced injury can be ameliorated by umbelliferone by suppressing oxidative stress and inflammatory responses. Umbelliferone pretreatment increased SOD and GSH levels and decreased MDA and MPO levels in kidney, heart and lung [8, 49]. In I/R-induced renal injury, ascorbic acid (AA) has been reported to increase GSH levels and decrease MDA levels [31]. Flavonoids such as umbelliferone and Myricetin, along with agents like apocynin and ascorbic acid, have shown comparable efficacy in mitigating oxidative stress in I/R injury. In our study, MYR administration decreased MDA and NO levels and increased GSH levels. These results indicate that MYR is an effective antioxidant agent in the fight against oxidative stress. Activation of NRF2/HO-1 signaling pathway is one of the mechanisms supporting the alleviation of oxidative stress and MYR seems to activate this pathway.

Inflammation is one of the main determinants of renal I/R injury. TNF-α, IL-1β, IL-6 and IL-15 levels were significantly increased in I/R group. Administration of MYR at doses of 50 and 100 mg/kg resulted in a significant decrease in these cytokine levels. Especially the decrease in TNF-α and IL-1β levels indicates that MYR stabilizes the immune response in kidney tissue by regulating inflammatory cytokine expression. Similarly, previous studies have shown that MYR suppresses inflammatory cytokines and inhibits TLR4/NF-κB pathway [50, 51]. Total flavonoids have been shown to reduce IL-1β, IL-6 and TNF-α mRNA levels [47]. Umbelliferone has been reported to suppress inflammation by reducing TNF-α and IL-6 levels [8]. Studies show that Myricetin, umbelliferone and other agents may suppress inflammation by reducing the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and decreasing MPO activity. This supports that inflammation plays an important role in the pathophysiology of renal I/R injury and that these agents may provide protective effects by targeting inflammation.

TLR4 triggers the inflammatory process by activating NF-κB through DAMPs. In the literature, it has been observed that the effects of TLR4 expression on cardiomyocyte injury are increased but suppressed by MYR treatment. These findings suggest that MYR inhibits TLR4 signaling pathway, limits NF-κB activation and suppresses inflammation. Dihydro-myricetin protects against LPS-induced cardiomyocyte injury through the TLR4/NF-kB pathway [52, 53]. It has been reported that TLR4 plays an important role in I/R injury and TLR4-deficient mice have smaller infarctions [53]. Another study reported that TLR4 expression in renal tubular cells increased after I/R injury [19].

Most flavonoids show anti-apoptotic effects in renal I/R injury. Luteolin pretreatment significantly decreased Bax (pro-apoptotic protein) expression and increased Bcl-2 (anti-apoptotic protein) expression [54]. Allicin treatment significantly decreased Bax, Caspase-3 and Cyt-C expression levels and significantly increased Bcl-2 expression level [53]. Apoptosis, necrosis and ferroptosis have an important place among cellular death mechanisms. In this study, it was shown that Caspase-3 levels increased, and Bcl-2 expression decreased in I/R group. MYR treatment attenuated apoptosis by downregulating Caspase-3 expression and supported cellular survival by increasing Bcl-2 levels. Myricetin has been shown to suppress the apoptotic process in previous studies and similar results were obtained in this studies [4, 55].

Our study shows that apoptosis, necrosis, and ferroptosis have an important place among cellular death mechanisms. In this study, it was shown that Caspase-3 levels increased, and Bcl-2 expression decreased in the I/R group. MYR administration decreased apoptosis by suppressing Caspase-3 expression and supported cellular survival by increasing Bcl-2 levels.

Conclusion

This study revealed that Myricetin suppresses inflammation, oxidative stress and apoptosis in renal I/R injury. These effects are mediated through NF-κB inhibition, NRF2 activation and modulation of TLR4 pathway. These findings suggest that Myricetin may be considered as a potential therapeutic agent in renal I/R injury. However, further research is needed for clinical trials and the effects of different doses. Also, future studies integrating mRNA and protein analyses will be essential to clarify whether observed protein-level changes are transcriptionally driven or influenced by alternative regulatory layers such as mRNA stability, translation efficiency, or post-translational modifications.

Study limitations

Our study has some limitations. First, different doses and administration times were not evaluated. More comprehensive studies are required for the long-term effects of Myricetin. Second, alternative mechanisms of inflammation and cellular death (necroptosis, ferroptosis) were not elaborated.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval Approval for this study was granted by the Local Animal Experimentation Ethics Committee at Kafkas University in Kars, with the approval number KAU-HADYEK 2024/118.

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