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NEPHROLOGY – ORIGINAL PAPER

Naringenin protects against renal ischemia–reperfusion injury following unilateral nephrectomy via modulation of oxidative stress and kisspeptin expression

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Abstract

Background Renal ischemia–reperfusion (IR) injury is a major cause of acute kidney injury, mediated by oxidative stress (OS), inflammation, and apoptosis. Naringenin (NRG), a citrus flavonoid, exerts antioxidant and anti-apoptotic actions, yet its role in renal kisspeptin signaling during IR injury remains unexplored. This study investigated the protective effects of NRG on renal IR injury in rats, with emphasis on OS, apoptosis, and kisspeptin expression.

Methods Thirty-two female Wistar albino rats were randomized into four groups: sham, IR, NRG/IR (100 mg/kg NRG administered 2 h before ischemia), and IR/NRG (NRG given at reperfusion). Following right nephrectomy and 60 min ischemia of the left kidney, reperfusion was allowed for 24 h. Serum (blood urea nitrogen, creatinine, albumin), tissue OS markers, histopathology, and immunohistochemical expression of kisspeptin, Bcl-2-associated X (Bax), and B cell lymphoma 2 (Bcl-2) were evaluated.

Results IR markedly increased malondialdehyde and reduced superoxide dismutase and catalase activities, accompanied by tubular degeneration, congestion, and dilatation. NRG treatment significantly reversed these biochemical and histological changes. Pre-ischemic NRG enhanced Bcl-2 immunoreactivity, while post-ischemic administration restored kisspeptin expression. Bax expression showed only mild non-significant increases. Notably, post-treatment provided superior recovery of kisspeptin signaling and structural preservation, whereas pre-treatment more effectively enhanced anti-apoptotic protein expression.

Conclusion NRG confers renoprotection against IR injury by reducing OS, preserving renal architecture, and modulating apoptosis-related proteins. A novel finding is the restoration of kisspeptin expression by NRG, particularly when administered at reperfusion, suggesting timing-dependent therapeutic potential.

Keywords Renal ischemia–reperfusion · Naringenin · Oxidative stress · Apoptosis · Kisspeptin

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Introduction

Renal ischemia, defined as a reduction or cessation of blood flow to the kidneys, is a critical event that can lead to acute kidney injury (AKI) and contribute to chronic kidney disease (CKD) and end-stage renal disease. This condition arises in various clinical contexts, including major surgery, shock, and kidney transplantation, and is a leading cause of morbidity and mortality in hospitalized patients [1–3].

The pathogenesis of renal ischemia involves a complex interplay of cellular and molecular mechanisms. Ischemia and subsequent reperfusion trigger cascades of cell death (necrosis, apoptosis, and regulated necrosis), inflammation, oxidative stress (OS), and microvascular dysfunction [2, 4,

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5]. Endothelial and epithelial cell injury, leukocyte infiltration, and the release of pro-inflammatory mediators further exacerbate tissue damage and impair renal function [1, 3]. Mitochondrial dysfunction and persistent tissue hypoxia after reperfusion are central to the development of both acute and chronic renal injury [6, 7].

In addition to its well-established role in reproductive regulation, kisspeptin and its receptor (KISS1R) are expressed in renal tissues and may contribute to the regulation of renal function and injury responses. Altered kisspeptin expression has been reported in experimental kidney injury models, suggesting a potential role in the pathogenesis of renal ischemia–reperfusion (IR) injury [8, 9].

Naringenin (NRG), a flavonoid abundantly found in citrus fruits, has emerged as a promising therapeutic agent due to its potent antioxidant and anti-apoptotic properties. Research demonstrates that NRG alleviates renal IR injury by activating the nuclear factor erythroid 2-related factor (Nrf2) transcription factor/heme oxygenase 1 (HO-1) signaling pathway, which enhances cellular antioxidant defenses and suppresses endoplasmic reticulum stress, thereby reducing both pyroptosis and apoptosis in renal tissues [10]. In experimental models, NRG administration leads to improved renal function, decreased tissue damage, and downregulation of pro-apoptotic markers such as Bcl-2-associated X (Bax) and caspase-3, while upregulating anti-apoptotic proteins like B cell lymphoma 2 (Bcl-2) [10, 11]. These multifaceted protective mechanisms position NRG as a potential candidate for mitigating renal injury associated with ischemic events.

The aim of this study is to investigate the effects of NRG, which has antioxidant and anti-apoptotic effects, on renal IR injury in rats via kisspeptin.

Materials and methods

Animals

This study utilized 32 female Wistar albino rats, aged 3 months and weighing between 250 and 300 g, sourced from the Inonu University Laboratory Animals Research Center. Female rats were used because kisspeptin expression is strongly modulated by estrogen-dependent mechanisms, making females a physiologically relevant model for studying kisspeptin-associated responses [12]. All animals were age-matched and housed under identical conditions to minimize variability related to hormonal status. They were housed in a controlled environment with a temperature of 21 ± 2 °C and humidity of 60 ± 5%, under a 12:12 h light:dark cycle. Rats were provided with a regular chow pellet diet and unrestricted access to tap water. Randomization was employed to allocate animals to several experimental groups and to gather and process data, with analysis conducted by scientists unaware of the treatment groups. All studies in this study adhered to the National Institutes of Health Animal Research standards and the ARRIVE standards [13]. The Ethics Committee on Animal Research (protocol no.: 2017/A-10) of the Faculty of Medicine, Inonu University, Malatya, Türkiye, accepted the study protocol on February 23, 2017. A straightforward randomization method was employed to assign the rats to groups, therefore mitigating bias in the experimental procedure.

Experimental design

Thirty-two Wistar albino rats were randomly divided into four groups:

Sham group: Only 60 min after right nephrectomy, 0.5 mL of dimethyl sulfoxide (DMSO) was administered intraperitoneally (i.p.) as a vehicle solvent.

IR group: Immediately before ending 60 min of ischemia after right nephrectomy, 0.5 mL DMSO was administered i.p. with the help of clamps on the left renal artery and vein, and left for 24 h of reperfusion with surgical closure.

NRG/IR group: 100 mg/kg NRG was administered as a single dose i.p. 2 h before right nephrectomy and left renal IR, and left to reperfuse for 24 h with surgical closure.

IR/NRG group: Immediately before the termination of 60 min of left kidney ischemia following right nephrectomy, a single dose of 100 mg/kg NRG was administered i.p., and the animals were allowed to undergo 24 h of reperfusion with surgical closure.

The renal IR protocol was adapted from our previously published unilateral nephrectomy-based renal IR studies [14, 15]. The NRG (CAS No.: 67604-48-2, Sigma-Aldrich, MO, USA) dose was selected based on previous studies demonstrating its renoprotective efficacy. Calis et al. reported that NRG administered at 100 mg/kg significantly attenuated renal injury in experimental hyperuricemia through suppression of xanthine oxidase activity, inflammation, apoptosis, and DNA damage, while enhancing antioxidant defenses [16]. Unilateral nephrectomy in renal IR models forces the injured kidney to carry total renal function, allowing systemic AKI to be measured, while also modifying renal hemodynamics and injury signaling in a timing-dependent manner [17, 18]. Renal ischemia was applied for 60 min followed by 24 h reperfusion, a commonly used acute renal IR model that consistently induces early AKI with prominent tubular injury and OS at 24 h, enabling detection of therapeutic effects while limiting confounding from later repair responses.

The abdominal region was disinfected with povidone-iodine 10% (Batticon antiseptic solution, ADEKA Pharmaceuticals, İstanbul, Türkiye) solution. Establishment of the experimental IR model and kidney tissue harvesting and blood collection was performed under intraperitoneal 75 mg/

kg ketamine (Ketasol 10% *w*/*v* injectable solution; Richter Pharma Ag, Wels, Austria) and 8 mg/kg xylazine (Xylazin-Bio 2% injectable solution, Bioveta PLC, Ivanovice na Hané, Czech Republic) anesthesia. The rats were then euthanized by surgical exsanguination. Subsequently, kidney tissue and blood specimens were collected from rats, followed by biochemical, histopathological and immunohistochemical studies. The kidneys were promptly excised, decapsulated, and bisected into two equal longitudinal segments. One portion was preserved in formalin for histopathological evaluation, while the remaining tissues were maintained at - 80 °C for biochemical investigation. Blood samples were obtained in tubes to assess blood urea nitrogen (BUN), creatinine (Cr) and albumin levels. Biochemical analyses [Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx)], histopathological assessments (congestion, infiltration, tubular degeneration and tubular dilatation) and immunohistochemical analysis (kisspeptin, Bax and Bcl-2) were conducted at the conclusion of the study protocol.

Biochemical analysis

Serum biochemical analysis

Serum concentrations of BUN, Cr, and albumin were analyzed at the Central Laboratory of Inonu University Turgut Özal Medical Center. BUN was measured using a urease–glutamate dehydrogenase enzymatic method (Roche Diagnostics, Mannheim, Germany) [19], Cr was determined by the kinetic Jaffe method (Roche Diagnostics, Mannheim, Germany) [20], and albumin levels were assessed by a bromocresol green colorimetric assay (Abbott Diagnostics, IL, USA). All analyses were carried out with an automated chemistry analyzer (e.g., Roche Cobas 8000 or equivalent system) [21], in accordance with the manufacturers' protocols. Serum albumin levels were measured as an additional indicator of renal functional status. Alterations in serum albumin may reflect changes in glomerular filtration, protein handling, and the systemic consequences of AKI [22].

Kidney tissue biochemical analysis

Kidney tissue was homogenized under ice isolation by adding phosphate buffer solution (PBS) (pH 7.4) until all tissue was broken down. The remaining homogenates were sonicated four times for 15 s at 30-s intervals. 1 mL of the resulting homogenate was set aside for MDA measurement. Following sonication, centrifugation was performed using a microcentrifuge. This yielded the supernatant, which would be used for enzyme activity and protein quantification. The supernatant samples were stored at − 80 °C in a deep freezer until measurement.

Esterbauer and Cheeseman's method was used [23]. The MDA amount was calculated in nanomoles per gram of tissue. SOD activity was measured using the nitroblue tetrazolium (NBT) reduction method described by Sun and colleagues and modified by Durak and colleagues [24, 25]. The results are expressed as U/mg protein. CAT activity was studied according to Aebi's method [26]. GSH concentration was measured according to the Beutler et al. method [27]. GSH level was expressed as µmol/g protein. GPx activity was studied according to the method of Paglia and colleagues [28]. The enzyme unit is the amount of micromoles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per unit time.

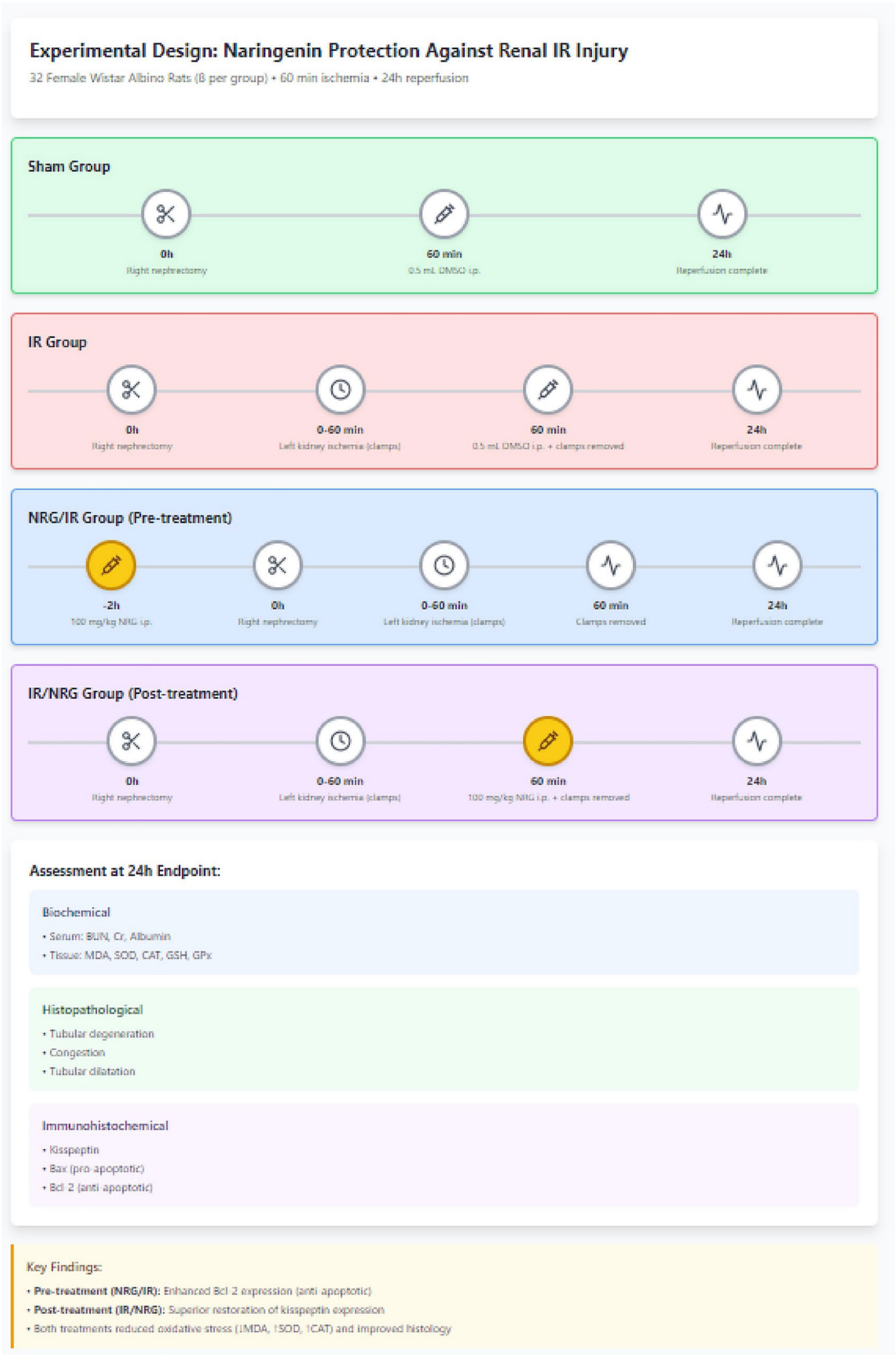
Histopathological analyses

The kidney tissue obtained at the end of the experiment was fixed in 10% formaldehyde. Sections 4–5 µm thick were taken from paraffin blocks prepared after tissue tracking procedures. Hematoxylin–eosin (H–E) staining was applied to the sections to determine the general morphological structure.

The kidney sections were examined for congestion (accumulation of blood within blood vessels), infiltration (accumulation of inflammatory cells), tubular degeneration (hydropic changes in tubular epithelial cells and shedding into the lumen), and tubular dilatation. Damage was scored according to severity as 0 (no change), 1 (mild damage), 2 (moderate damage), and 3 (severe damage), and 10 randomly selected areas were evaluated. Analyses were performed using a Leica DFC-280 research microscope and the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Immunohistochemical analyses

For immunohistochemical analyses, sections that had undergone deparaffinization and rehydration were placed in a pressure cooker and boiled in 0.01 M citrate (pH 6.0) for 15–20 min. To block endogenous peroxidase enzyme activity, sections were treated with 3% hydrogen peroxide for 12 min. Sections washed with PBS were treated with protein block (ultra V block) for 5 min. Sections were then incubated with primary antibody. For immunohistochemical analyses, the following primary antibodies were used: Kisspeptin rabbit polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. BS-0749R; dilution: 1:50), anti-Bax (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-7480; dilution: 1:100), and anti-Bcl-2 (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-7382; dilution: 1:100). The tissues washed with PBS were treated with biotinylated secondary antibody at 37 °C for 10 min. After this process, the sections were incubated with streptavidin


Experimental Design: Naringenin Protection Against Renal IR Injury
32 Female Wistar Albino Rats (8 per group) • 60 min ischemia • 24h reperfusion
Sham Group
0h
Right nephrectomy
60 min
0.5 mL DMSO i.p.
24h
Reperfusion complete
IR Group
0h
Right nephrectomy
0-60 min
Left kidney ischemia (clamps)
60 min
0.5 mL DMSO i.p. + clamps removed
24h
Reperfusion complete
NRG/IR Group (Pre-treatment)
-2h
100 mg/kg NRG i.p.
0h
Right nephrectomy
0-60 min
Left kidney ischemia (clamps)
60 min
Clamps removed
24h
Reperfusion complete
IR/NRG Group (Post-treatment)
0h
Right nephrectomy
0-60 min
Left kidney ischemia (clamps)
60 min
100 mg/kg NRG i.p. + clamps removed
24h
Reperfusion complete
Assessment at 24h Endpoint:
Biochemical
• Serum: BUN, Cr, Albumin
• Tissue: MDA, SOD, CAT, GSH, GPx
Histopathological
• Tubular degeneration
• Congestion
• Tubular dilatation
Immunohistochemical
• Kisspeptin
• Bax (pro-apoptotic)
• Bcl-2 (anti-apoptotic)
Key Findings:
• Pre-treatment (NRG/IR): Enhanced Bcl-2 expression (anti-apoptotic)
• Post-treatment (IR/NRG): Superior restoration of kisspeptin expression
• Both treatments reduced oxidative stress (↓MDA, ↑SOD, ↑CAT) and improved histology

◂**Fig. 1** Experimental design and protective effects of naringenin (NRG) against renal ischemia–reperfusion (IR) injury. Biochemical assessments included serum blood urea nitrogen (BUN), creatinine (Cr), and albumin, as well as tissue malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GPx). Immunohistochemical analyses included kisspeptin, Bcl-2-associated X protein (Bax), and B cell lymphoma 2 (Bcl-2). Oxidative stress (OS) is indicated in the summary of key findings

peroxidase at 37 °C for 10 min. The sections treated with chromogen were then stained with hematoxylin and sealed with a water-based sealant.

Staining was semi-quantitatively scored based on the prevalence (0: 0–25%, 1: 26–50%, 2: 51–75%, 3: 76–100%) and intensity (0: absent, + 1: mild, + 2: moderate, + 3: severe) of immunoreactivity. The total staining score was calculated as prevalence × severity [29].

Statistical analysis

Statistical analyses were performed using the SPSS for Windows version 18.0 software package. Where appropriate, data were summarized as median (min–max) or interquartile range (IQR). For intergroup comparisons, nonparametric methods were preferred based on the distribution characteristics of the data. First, the Kruskal–Wallis test was applied to compare the four groups. To determine which groups were responsible for the statistically significant differences in parameters, the Conover post-hoc pairwise comparison test was used. For some biochemical parameters, pairwise group comparisons were additionally performed using the Mann–Whitney U test. In all statistical tests, the significance level was set at $p < 0.05$.

Results

Experimental design and NRG's effects on OS, apoptosis, and kisspeptin summarized in graphical abstract (Fig. 1).

Effects of NRG on serum renal function biomarkers

Serum BUN levels differed significantly among the experimental groups ($p < 0.001$). The IR group exhibited markedly elevated BUN levels compared with the sham group ($p = 0.007$). Although BUN levels were numerically lower in the NRG/IR group than in the IR group, this reduction was not statistically significant. Similarly, no significant difference was observed between the IR and IR/NRG groups. NRG/IR and IR/NRG groups continued to exhibit significantly higher BUN levels than the sham group ($p = 0.003$ and $p = 0.002$, *respectively*) (Table 1).

Serum Cr levels differed significantly among the experimental groups ($p = 0.002$). The IR group exhibited significantly higher Cr levels than the sham group ($p = 0.007$). Although Cr levels were numerically lower in the NRG/IR group than in the IR group, this difference was not statistically significant. Similarly, no significant difference was observed between the IR and IR/NRG groups. NRG/IR and IR/NRG groups remained significantly different from the sham group ($p = 0.003$ and $p = 0.001$, *respectively*) (Table 1).

Serum albumin levels were comparable among the experimental groups, and no statistically significant difference was observed ($p = 0.178$). Although albumin levels tended to be lower in the IR group and higher in the NRG-treated groups compared with the sham group, these variations did not reach statistical significance (Table 1).

Effects of NRG on renal OS markers

Renal tissue MDA levels differed significantly among the experimental groups ($p < 0.001$). The IR group exhibited significantly higher MDA levels than the sham group ($p = 0.001$). Both NRG/IR and IR/NRG treatment significantly reduced MDA levels compared with the IR group ($p = 0.007$ and $p = 0.001$). No significant differences were observed between the sham, NRG/IR, and IR/NRG groups (Table 2).

Renal tissue SOD levels differed significantly among the experimental groups ($p < 0.001$). The IR group exhibited significantly lower SOD levels than the sham group ($p = 0.009$). Both NRG/IR and IR/NRG treatment significantly increased SOD levels compared with the IR group ($p = 0.001$ and $p = 0.001$). No significant differences were observed between the sham, NRG/IR, and IR/NRG groups (Table 2).

Renal tissue CAT levels differed significantly among the experimental groups ($p = 0.001$). The IR group exhibited significantly lower CAT levels than the sham group ($p = 0.001$). Both NRG/IR and IR/NRG treatment significantly increased CAT levels compared with the IR group ($p = 0.007$ and $p = 0.002$). No significant differences were observed between the sham, NRG/IR, and IR/NRG groups (Table 2).

Renal tissue GSH levels did not differ significantly among the experimental groups ($p = 0.251$). Although GSH levels were numerically lower in the IR group and higher in both NRG-treated groups compared with the sham group, these differences did not reach statistical significance (Table 2).

Renal tissue GPx levels differed significantly among the experimental groups ($p = 0.002$). The sham group exhibited significantly higher GPx levels than the IR, NRG/IR, and IR/NRG groups ($p = 0.007$, $p = 0.002$ and $p = 0.006$, *respectively*). No significant differences were observed among the IR, NRG/IR, and IR/NRG groups (Table 2).

Table 1 Effects of NRG on serum renal function biomarkers following renal IR injury

	Groups				*p* value
	Sham	IR	NRG/IR	IR/NRG	
Blood urea nitrogen (mg/dL)	23.7 (9.35)	98.67[a] (26.53)	78.69[a] (18.25)	88.65[a] (31.62)	**< 0.001**
Creatinine (mg/dL)	0.63 (0.06)	1.27[a] (0.58)	1.14[a] (0.5)	1.5[a] (0.56)	**0.002**
Albumin (g/dL)	1.05 (0.22)	0.95 (0.1)	1.15 (0.18)	1.1 (0.2)	0.178

Bold values indicate statistically significant differences ($p < 0.05$)

Data are shown as medians (interquartile range)

NRG naringenin, *IR* ischemia–reperfusion

a: There is a statistically significant difference compared to the sham group ($p < 0.05$)

Table 2 Effects of NRG on renal OS markers after IR injury

	Group				*p* value
	Sham	IR	NRG/IR	IR/NRG	
Malondialdehyde (nmol/g tissue)	25.96 (11.47)	46[a] (16.82)	27.2[b] (10.17)	23.82[b] (10.76)	**< 0.001**
Superoxide dismutase (U/mg protein)	0.57 (0.17)	0.42[a] (0.05)	0.52[b] (0.02)	0.60[b] (0.20)	**< 0.001**
Catalase (K/g protein)	21.52 (5.03)	10.68[a] (5.98)	22.15[b] (7.25)	20.62[b] (4.27)	**0.001**
Glutathione (µmol/g tissue)	10.84 (1.59)	9.7 (1.59)	12.26 (2.59)	11.93 (2.13)	0.251
Glutathione peroxidase (U/mg protein)	58.13 (22.18)	39.83[a] (8.32)	38.09[a] (4.32)	41.98[a] (9.02)	**0.002**

Bold values indicate statistically significant differences ($p < 0.05$)

Data are shown as medians (interquartile range)

NRG naringenin, *IR* ischemia–reperfusion

a: There is a statistically significant difference compared to the sham group ($p < 0.05$)

b: There is a statistically significant difference compared to the IR group ($p < 0.05$)

Effects of NRG on renal histopathological injury

Histopathological examination revealed significant alterations in tubular dilatation, congestion, and tubular degeneration following renal IR injury (Table 3, Fig. 2).

Tubular dilatation scores were significantly higher in the IR group than in the sham group ($p < 0.001$). Tubular dilatation was significantly reduced in both the NRG/IR and IR/NRG groups compared with the IR group ($p = 0.001$ and $p < 0.001$, respectively). No significant difference was observed between the NRG/IR and IR/NRG groups ($p = 0.367$).

Congestion scores were significantly increased in the IR group compared with the sham group ($p < 0.001$). While no significant reduction was observed in the NRG/IR group compared with the IR group ($p = 0.566$), congestion scores were significantly lower in the IR/NRG group than in the IR group ($p = 0.013$). In addition, congestion was significantly lower in the IR/NRG group than in the NRG/IR group ($p = 0.045$).

Tubular degeneration scores were significantly higher in the IR group than in the sham group ($p = 0.002$). No significant difference was detected between the IR and NRG/IR groups ($p = 0.282$), whereas tubular degeneration was significantly reduced in the IR/NRG group compared with the IR group ($p < 0.001$).

No statistically significant differences were observed among the groups with respect to inflammatory cell infiltration (all $p > 0.05$).

Effects of NRG on renal kisspeptin and apoptosis-related protein expression

Renal kisspeptin immunoreactivity differed significantly among the experimental groups. The IR group exhibited significantly lower kisspeptin immunoreactivity than the sham group ($p < 0.001$). Similarly, kisspeptin

Table 3 Effects of NRG on semiquantitative renal histopathological injury scores

	Tubular dilation	Infiltration	Congestion	Tubular degeneration
Sham group	0.0 (0.0–2.0)	0.0 (0.0–3.0)	0.0 (0.0–2.0)	1.0 (0.0–3.0)
IR group	2.0[a] (0.0–3.0)	0.0 (0.0–3.0)	1.0[a] (0.0–3.0)	2.0[a] (0.0–3.0)
NRG/IR group	1.0[a,b] (0.0–3.0)	0.0 (0.0–2.0)	1.0[a] (0.0–3.0)	1.0 (0.0–3.0)
IR/NRG group	1.0[b] (0.0–3.0)	0.0[a] (0.0–2.0)	1.0[a,b,c] (0.0–3.0)	1.0[b,c] (0.0–3.0)
p-values for pairwise comparisons between groups				
Sham–IR	0.000	0.612	0.000	0.002
Sham–NRG/IR	0.005	0.236	0.000	0.057
Sham–IR/NRG	0.064	0.022	0.008	0.088
IR–NRG/IR	0.001	0.478	0.566	0.282
NRG/IR–IR/NRG	0.367	0.332	0.045	0.001
IR–IR/NRG	0.000	0.076	0.013	0.000

Data are shown as median (min–max)

NRG naringenin, *IR* ischemia–reperfusion

a: Statistically different from the sham group ($p < 0.05$)

b: Statistically different from the IR group ($p < 0.05$)

c: Statistically different from the NRG/IR group ($p < 0.05$)

immunoreactivity remained significantly reduced in the NRG/IR group compared with the sham group ($p < 0.001$), with no significant difference between the IR and NRG/IR groups ($p = 0.372$). In contrast, the IR/NRG group demonstrated significantly higher kisspeptin immunoreactivity than the IR group ($p = 0.025$). No significant difference was observed between the NRG/IR and IR/NRG groups ($p = 0.161$), whereas kisspeptin immunoreactivity in the IR/NRG group remained significantly lower than that in the sham group ($p = 0.001$) (Table 4, Fig. 3).

No statistically significant differences in renal Bax immunoreactivity were observed among the experimental groups. Although Bax immunoreactivity was numerically higher in the IR group than in the sham group, this difference did not reach statistical significance ($p = 0.484$). Similarly, no significant differences were detected between the IR and NRG/IR groups ($p = 0.123$), the IR and IR/NRG groups ($p = 0.106$), or the NRG/IR and IR/NRG groups ($p = 0.953$). Comparisons of the NRG/IR and IR/NRG groups with the sham group also revealed no statistically significant differences ($p = 0.313$ and $p = 0.282$, respectively) (Table 4, Fig. 4).

Renal Bcl-2 immunoreactivity differed significantly among the experimental groups. No significant difference was observed between the sham and IR groups ($p = 0.797$). However, Bcl-2 immunoreactivity was significantly higher in the NRG/IR group than in both the sham and IR groups ($p = 0.004$ and $p = 0.015$, respectively). In contrast, Bcl-2 immunoreactivity in the IR/NRG group did not differ significantly from that in the sham or IR groups ($p = 0.906$ and $p = 0.991$, respectively). Furthermore, Bcl-2 immunoreactivity was significantly lower in the IR/NRG group than in the NRG/IR group ($p = 0.014$) (Table 4, Fig. 5).

Discussion

In this study, we investigated the effects of NRG administered either before ischemia or at the onset of reperfusion in a rat model of renal IR injury. The principal findings demonstrate that renal IR induced marked OS, tubular damage, and apoptotic signaling, accompanied by a reduction in renal kisspeptin immunoreactivity. NRG treatment significantly attenuated these pathological changes; however, the protective profile was strongly dependent on the timing of administration. Pre-ischemic NRG predominantly enhanced anti-apoptotic signaling, as reflected by increased Bcl-2 expression, whereas post-ischemic NRG was more effective in restoring renal kisspeptin expression during early reperfusion. Direct comparison between pre- and post-treatment groups further revealed a phase-dependent divergence in molecular responses, supporting the existence of a dual-phase protective window for NRG in renal IR injury.

An important methodological aspect of the present study is the use of unilateral nephrectomy in conjunction with renal IR. Removal of the contralateral kidney was intentionally performed to eliminate compensatory renal function, thereby ensuring that systemic functional parameters and tissue-level outcomes accurately reflected injury and recovery in the ischemic kidney [30]. In addition to increasing the sensitivity of biochemical and histopathological assessments, unilateral nephrectomy modifies renal hemodynamics and injury-related signaling pathways, which may influence the magnitude and pattern of IR injury [31]. This approach also enhances the translational relevance of the model by mimicking clinical scenarios characterized by reduced renal reserve, such as kidney transplantation or partial nephrectomy.

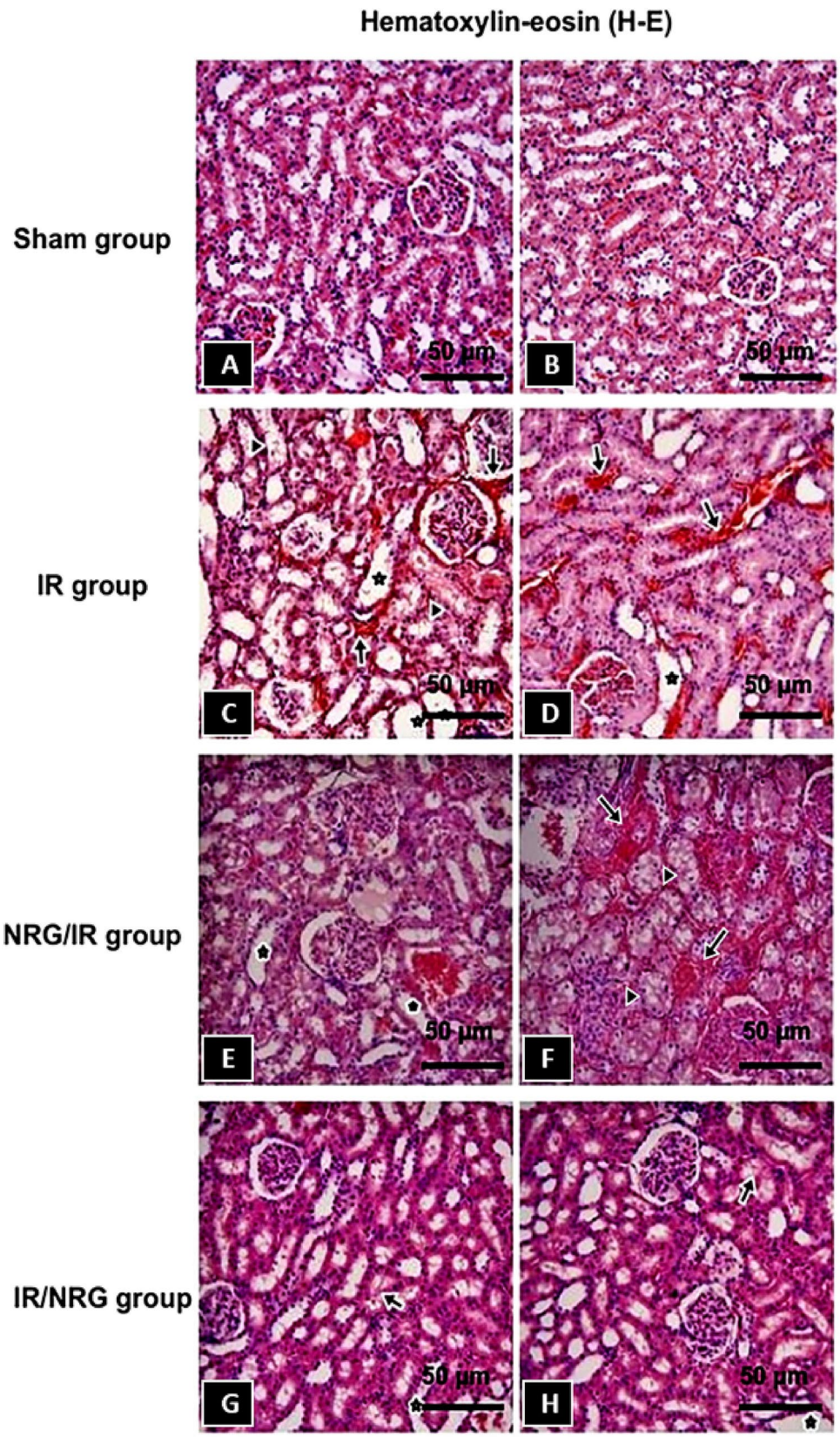


Fig. 2 Representative histopathological changes in renal tissue following ischemia–reperfusion (IR) injury and naringenin (NRG) treatment. The sham group shows the normal histological appearance of kidney tissue with H&E staining (**A**, **B**). The IR group shows increased tubular dilatation, and increase in congestion and tubular degeneration is observed (**C**, **D**). The NRG/IR group shows a decrease in the tubular dilatation (**E**, **F**). The IR/NRG group shows tubular dilatation similar to the sham group (**G**, **H**). It is noteworthy that the increased congestion in the IR and NRG/IR groups is significantly reduced in this group (**G**, **H**) (star: tubular dilatation, arrow: congestion, arrowhead: tubular degeneration) H/E

Table 4 Effects of NRG on renal kisspeptin, Bax, and Bcl-2 immunoreactivity scores

	Kisspeptin	Bax	Bcl-2
Sham group	8.0 (1.0–16.0)	3.0 (0.0–9.0)	4.0 (0.0–12.0)
IR group	3.5[a] (0.0–12.0)	4.0 (0.0–12.0)	4.0 (0.0–12.0)
NRG/IR group	4.0[a] (0.0–9.0)	3.0 (0.0–12.0)	6.0[a,b] (0.0–12.0)
IR/NRG group	6.0[a,b] (0.0–12.0)	3.0 (0.0–12.0)	4.0[c] (0.0–12.0)
p-values for pairwise comparisons between groups			
Sham–IR	0.000	0.484	0.797
Sham–NRG/IR	0.000	0.313	0.004
Sham–IR/NRG	0.001	0.282	0.906
IR–NRG/IR	0.372	0.123	0.015
NRG/IR–IR/NRG	0.161	0.953	0.014
IR–IR/NRG	0.025	0.106	0.991

Data are shown as median (min–max)

Bax Bcl-2-associated X, *Bcl-2* B cell lymphoma 2, *NRG* naringenin, *IR* ischemia–reperfusion

a: Statistically different from the sham group ($p < 0.05$)

b: Statistically different from the IR group ($p < 0.05$)

c: Statistically different from the NRG/IR group ($p < 0.05$)

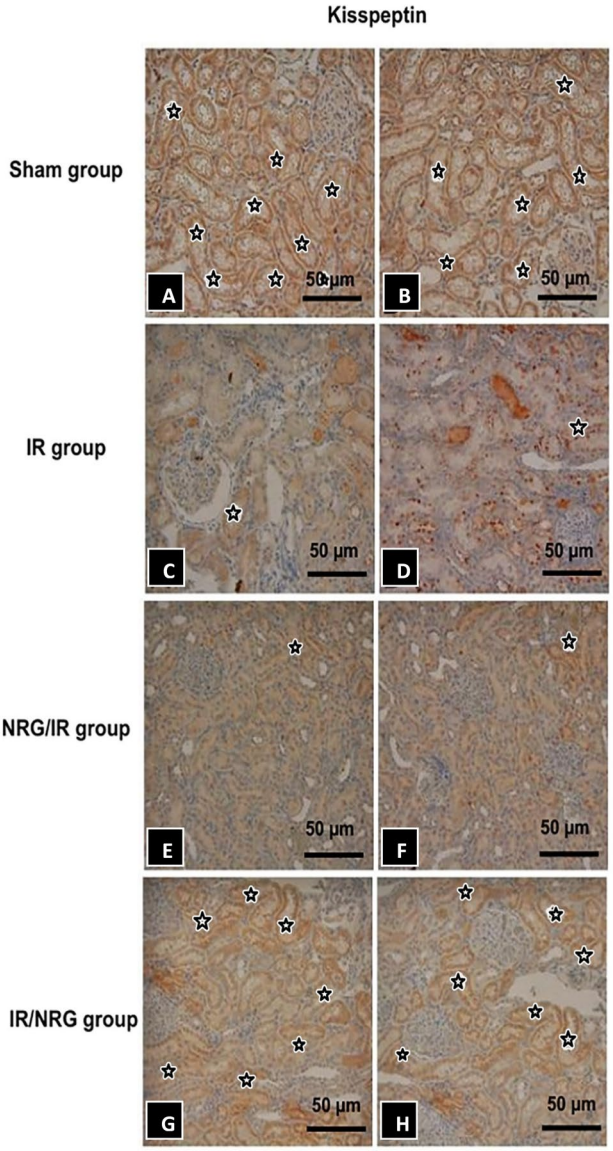


Fig. 3 Representative immunohistochemical staining for renal kisspeptin following ischemia–reperfusion (IR) injury and naringenin (NRG) treatment. In the sham group, kisspeptin reactivity is observed in the renal tubules (**A**, **B**). In the IR group, a decrease in kisspeptin immunoreactivity in the tubules is noted (**C**, **D**). In the NRG/IR group, a decrease in immunoreactivity in the tubules is observed (**E**, **F**). In the IR/NRG group, an increase in immunoreactivity in the tubules is observed (**G**, **H**) (stars: kisspeptin immunoreactivity)

Renal IR injury remains a major contributor to AKI, characterized by OS, inflammation, and apoptosis that culminate in tubular epithelial cell dysfunction and structural damage [1–3]. In our study, the IR model induced significant biochemical, histopathological, and molecular alterations in the kidney tissue of rats, consistent with previous findings [4, 5, 32]. Notably, treatment with NRG either prior to or immediately following ischemia conferred substantial renoprotection, particularly through modulation of OS markers and apoptosis-related proteins. The 24-h endpoint was selected to interrogate early AKI-phase mechanisms (OS and acute tubular injury). Later reperfusion time points (48–72 h) may better capture repair/fibrosis trajectories and should be evaluated in future work [33]. This design choice allowed isolation of acute oxidative and tubular injury pathways without confounding effects from subsequent repair processes.

Lipid peroxidation, as reflected by elevated MDA levels, was significantly increased in the IR group, indicating heightened OS. This is in line with prior studies reporting increased MDA in renal IR injury due to reactive oxygen species (ROS)-mediated membrane damage [5, 10, 11]. Both pre- and post-treatment with NRG significantly reduced MDA levels, restoring them to near-sham values.

Our findings are consistent with recent studies highlighting the central role of OS in the pathogenesis of renal IR injury. Karimi et al. demonstrated that attenuation of OS, reflected by reduced oxidative burden and improved antioxidant capacity, was associated with improved renal function and histopathological outcomes following renal IR injury [34]. Similarly, the reduction in MDA levels and restoration of antioxidant enzyme activities observed in the present study support the importance of redox balance in limiting IR-induced renal damage. SOD and CAT activities were markedly reduced in the IR group and significantly restored by NRG treatment, indicating enhancement of endogenous antioxidant defenses. Similar findings have been reported in previous studies demonstrating that

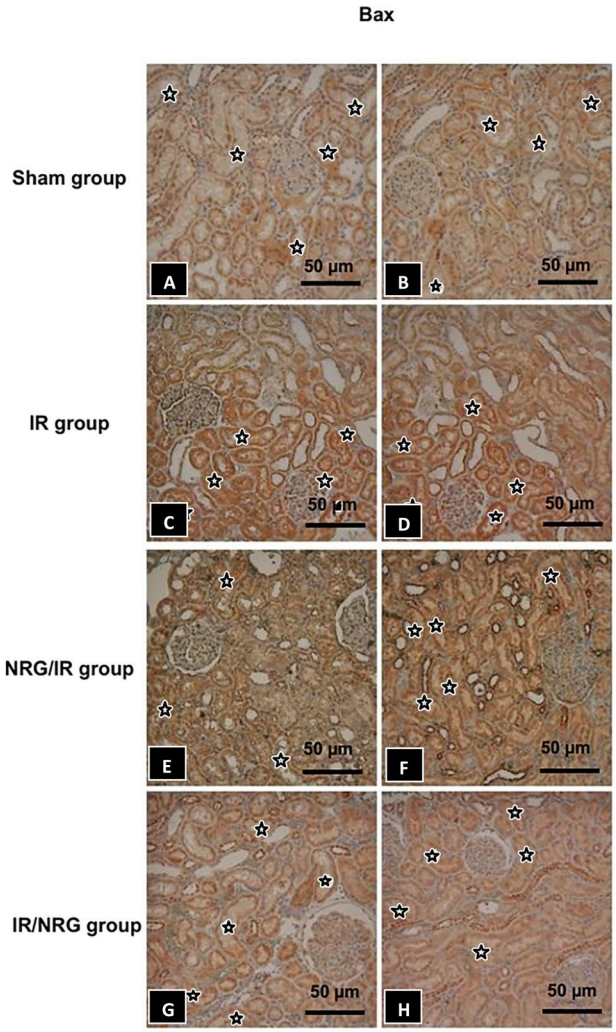


Fig. 4 Representative Bcl-2-associated X (Bax) immunoreactivity in renal tissue following ischemia–reperfusion (IR) injury and naringenin (NRG) treatment. Immune reactivity in the tubules is observed with Bax immunostaining in the sham group (**A**, **B**). Immune reactivity in the tubules with Bax immunostaining in the IR group is observed to be similar to that in the sham group (**C**, **D**). The NRG/IR group shows immunoreactivity in the tubules similar to that of the IR and sham groups (**E**, **F**). The IR/NRG group shows immunoreactivity in the tubules similar to that of the IR and sham groups (**G**, **H**) (stars: Bax immunoreactivity)

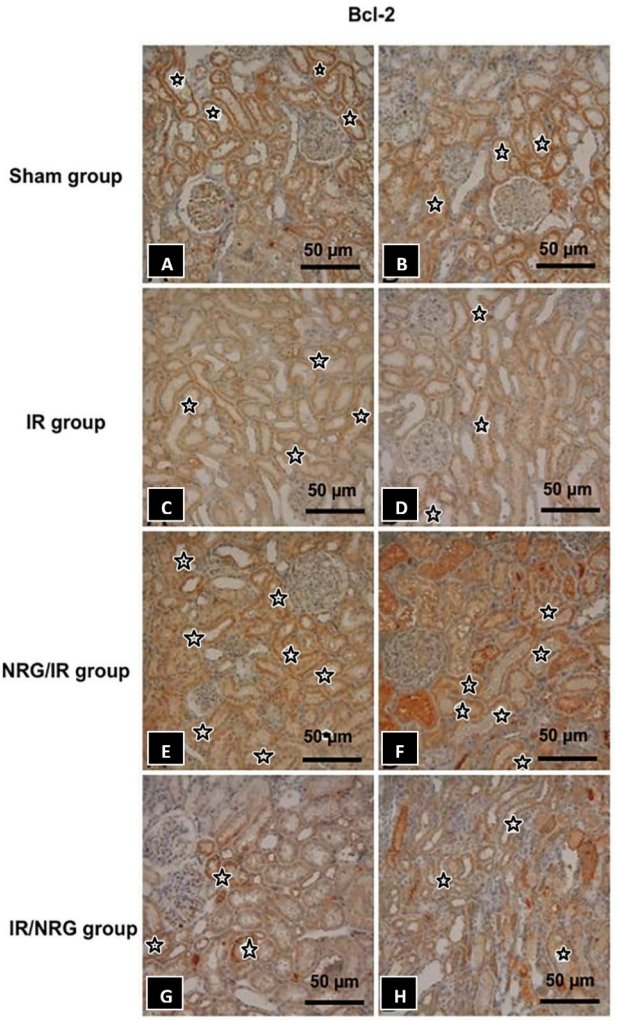


Fig. 5 Representative B cell lymphoma 2 (Bcl-2) immunoreactivity in renal tissue following ischemia–reperfusion (IR) injury and naringenin (NRG) treatment. Immunoreactivity in the tubules is observed with Bcl-2 immunostaining in the sham group (**A**, **B**). Immunoreactivity in the tubules is observed to be similar to that in the sham group in the IR group (**C**, **D**). The NRG/IR group shows increased immunoreactivity in the tubules (**E**, **F**). The IR/NRG group shows immunoreactivity in the tubules similar to that of the IR group (**G**, **H**) (star: Bcl-2 immunoreactivity)

NRG improves antioxidant enzyme capacity and attenuates ROS-mediated tissue injury [10, 35, 36].

The antioxidant effects observed in the present study are in line with previous reports involving structurally related citrus flavonoids. Amini et al. demonstrated that naringin (NAR), the glycoside precursor of NRG, attenuated renal IR-induced OS by reducing lipid peroxidation and restoring antioxidant enzyme activities. The authors further reported enhancement of Nrf2-mediated antioxidant defense mechanisms [37]. Although NAR and NRG are distinct compounds, their shared flavanone structure and overlapping biological activities suggest that attenuation of OS may represent a common mechanism underlying their renoprotective effects.

Regarding apoptosis, although Bax immunoreactivity showed a non-significant increase in the IR group, Bcl-2 expression was significantly elevated only in the NRG/IR group. This selective upregulation of the anti-apoptotic protein Bcl-2 aligns with previous studies demonstrating that NRG shifts the Bax/Bcl-2 ratio toward cell survival [11, 38]. The lack of significant Bcl-2 induction in the IR/NRG group, despite functional and histological improvements, implies that NRG's post-ischemic benefits may rely more

on antioxidant and anti-inflammatory mechanisms—or kisspeptin-mediated pathways—than on classical anti-apoptotic signaling. The immunohistochemical analysis demonstrated a significant decrease in Bcl-2 expression in the IR group, with a concomitant mild increase in pro-apoptotic Bax staining, although the latter did not reach statistical significance. This pattern implies an imbalance between pro- and anti-apoptotic signaling in IR injury. NRG pre-treatment significantly upregulated Bcl-2 expression, indicating that its protective effect may involve the inhibition of mitochondrial apoptosis pathways [10, 11, 39]. This aligns with previous work showing that NRG attenuates renal apoptosis by modulating Bcl-2/Bax and caspase-3 activity [11, 40, 41]. Amini et al. reported that NAR reduced renal IR-induced apoptosis through downregulation of Bax and caspase-3 expression and upregulation of Bcl-2 [11]. While the present study investigated NRG rather than NAR, both flavonoids have been reported to exert anti-apoptotic and antioxidant effects in experimental models of tissue injury. Therefore, the findings of the current study further support the potential role of citrus-derived flavanones in limiting IR-induced renal damage.

The exclusive use of female rats should be considered in the context of kisspeptin biology. Kisspeptin is highly sensitive to estrogenic regulation, and females display greater kisspeptin signaling plasticity [42]. While hormonal modulation may contribute to absolute kisspeptin levels, all experimental groups shared the same hormonal background; therefore, the observed intergroup differences primarily reflect treatment effects. Nonetheless, sex-specific mechanisms cannot be excluded, and future studies incorporating male animals, estrous cycle stratification, or hormonal manipulation will be informative.

One of the novel findings of this study is the observed modulation of renal kisspeptin expression following IR and NRG treatment. Kisspeptin, traditionally recognized for its role in reproductive neuroendocrinology, is also expressed in the kidney and has been implicated in renal development, hemodynamics, and injury response. Previous studies have demonstrated altered renal kisspeptin expression in both acute and chronic kidney injury models, suggesting a potential role for kisspeptin signaling in renal pathophysiology [8, 9]. Existing studies on NRG's renal effects focus on anti-inflammatory, antioxidant, and anti-apoptotic mechanisms, without mention of kisspeptin pathways [10, 43]. Our immunohistochemical analysis revealed a significant downregulation of kisspeptin immunoreactivity in the IR group compared to sham group, consistent with prior evidence linking reduced kisspeptin signaling to renal dysfunction [8]. Intriguingly, post-ischemic NRG administration restored kisspeptin expression to near-sham levels, whereas pre-treatment did not. This suggests that NRG's ability to preserve or restore kisspeptin signaling may be contingent on the timing of administration and may contribute to its renoprotective effects during the reperfusion phase. Recent studies suggest that kisspeptin and its receptor KISS1R are expressed in renal tissues and may influence tubular integrity and inflammatory responses [8, 9]. Our results are consistent with Kudaibergenova et al. [8], who demonstrated a downregulation of kisspeptin in experimental renal IR injury, and suggest that NRG may restore kisspeptin signaling as part of its renoprotective effect.

Emerging evidence suggests that kisspeptin signaling may interact with OS-related pathways in various tissues. Experimental studies have shown that OS can suppress KISS1/KISS1R expression, whereas restoration of cellular redox balance may normalize kisspeptin signaling [44, 45]. In addition, kisspeptin has been reported to influence mitochondrial function, inflammatory responses, and cellular survival pathways, all of which are closely linked to oxidative injury [46]. In the present study, the reduction in kisspeptin immunoreactivity observed in the IR group occurred in parallel with increased lipid peroxidation and impaired antioxidant enzyme activities. Conversely, restoration of kisspeptin expression in the IR/NRG group was accompanied by reduced MDA levels and improved antioxidant defenses. Although these findings do not establish a causal relationship, they suggest a potential association between kisspeptin signaling and OS regulation during renal IR injury.

Kidney-focused work has shown that kisspeptin and KISS1R expression are altered in chronic renal failure, with changes in mRNA and protein levels in renal tubular, collecting duct and vascular smooth muscle cells, suggesting a role in kidney pathophysiology but without direct apoptosis signaling data [47]. A more recent review similarly notes that kisspeptin/KISS1R expression is dysregulated in chronically impaired kidneys, but again does not map it onto apoptotic signaling hierarchies [48]. In CKD-uremic cardiomyopathy models, kisspeptin agonism or antagonism changes cardiac expression of Bax/Bcl-2/caspase-7, consistent with kisspeptin signaling affecting apoptosis-related genes in vivo [9, 49]. Expression changes of kisspeptin/KISS1R have been reported in CKD kidneys; however, no studies have clearly demonstrated that Bax/Bcl-2/caspases signaling occurs downstream of kisspeptin in renal cells [47, 48].

Taken together, the parallel reduction of OS markers and restoration of kisspeptin immunoreactivity following NRG treatment suggests that kisspeptin signaling may participate in the renal response to oxidative injury. Although causality cannot be established from the present study, these findings support the hypothesis that modulation of kisspeptin pathways may represent one component of the renoprotective actions of NRG during IR injury.

Notably, only the IR/NRG group showed a statistically significant improvement in histopathological parameters

compared to the untreated IR group, suggesting that NRG may be particularly effective when administered at the onset of reperfusion. This timing-dependent efficacy is clinically relevant, as many therapeutic interventions in AKI are initiated after the ischemic insult has already occurred. Histopathological findings further supported the biochemical and immunohistochemical results. The IR group showed marked tubular degeneration, congestion, and dilation—hallmarks of acute tubular injury. These lesions were significantly attenuated in both NRG treatment groups, confirming its structural protective role, in line with prior experimental observations [11, 38, 50, 51].

In contrast to previous studies that mainly emphasized the suppression of endoplasmic reticulum stress, pyroptosis, and apoptosis via Nrf2/HO-1 signaling [10]. Our study introduces two novel aspects. First, by using both pre- and post-ischemic treatment protocols in a rat IR model, we provide evidence for the timing-dependent efficacy of NRG. Second, we show for the first time that NRG can modulate renal kisspeptin expression, particularly restoring its levels when administered at reperfusion. These findings extend the known antioxidative and anti-apoptotic actions of NRG and highlight kisspeptin signaling and treatment timing as distinct mechanistic contributions of this study.

The divergent molecular profiles observed between pre- and post-ischemic NRG administration suggest a dual-phase protective window. Pre-treatment appears to primarily engage anti-apoptotic defenses, likely by enhancing cellular resistance prior to ischemic stress. Conversely, post-treatment during reperfusion preferentially restores kisspeptin signaling, which may reflect activation of recovery-associated pathways rather than direct apoptosis suppression. This temporal dissociation underscores that the timing of NRG delivery critically influences its dominant protective mechanism.

The histopathological evaluation was performed on randomly selected kidney sections without separate quantitative analysis of cortical and medullary regions. Therefore, although medullary structures were present in the examined sections, the current study was not specifically designed to assess regional differences between the cortex and outer medulla. Future studies incorporating region-specific scoring and dedicated medullary sampling may provide additional insight into the spatial distribution of renal IR injury.

Conclusion

Collectively, our findings support the multifaceted protective profile of NRG in renal IR injury. A novel observation is that NRG—particularly when administered at reperfusion—restored renal kisspeptin immunoreactivity in parallel with structural and oxidative improvements, suggesting a potential link that should be validated by KISS1R-targeted mechanistic experiments. However, further studies are needed to elucidate the precise molecular interplay between NRG and kisspeptin, and to validate these findings in higher-order models or human tissues. The use of a unilateral nephrectomy-based IR model strengthened the interpretability of the findings by eliminating contralateral compensation and allowing clearer evaluation of NRG's renoprotective effects.

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Author contributions Leyla Sahin Buyukkorkmaz: Conceptualization, Visualization, Data curation, Writing—Original draft preparation, Writing—Reviewing and Editing. Seda Tasdemir: Supervision, Investigation, Data curation, Validation, Methodology. Onural Ozhan: Investigation, Methodology, Visualization. Isil Eranil: Investigation, Methodology, Visualization. Kevser Tanbek: Investigation, Methodology, Visualization. Nigar Vardi: Investigation, Methodology, Visualization. Harika Gozde Gozukara Bag: Investigation, Data curation, Validation, Investigation, Methodology. Ahmet Acet: Supervision, Writing—Reviewing and Editing. Hakan Parlakpinar: Supervision, Writing—Reviewing and Editing.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Ethical approval (Protocol no.: 2017/A-10) was obtained from the Experimental Animal Ethics Committee of Inonu University Faculty of Medicine in 2017.

Clinical trial Not applicable.

Consent to publish Not applicable.

Consent to participate Not applicable.

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