

Renoprotective Effect of S14G-Humanin on Renal Ischemia/Reperfusion Injury by Activation of STAT3 and ERK 1/2 Signal Transduction Pathways in Rats

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Abstract

Renal ischemia/reperfusion (I/R) injury leads to acute tubular necrosis and renal failure, triggering pathological mechanisms including inflammation, reactive oxygen species generation, apoptosis, and mitochondrial dysfunction. The mitochondrial peptide Humanin (HN), known to possess anti-apoptotic and anti-inflammatory properties, has been shown to counteract oxidative stress and restore mitochondrial function. This study aimed to investigate the effects of HN on renal I/R injury.

Sprague-Dawley male rats were divided into four groups (n = 48): 1.Sham, 2.I/R, 3.HN-Sham, 4.HN-I/R. In I/R groups, renal artery ligation was performed for 45 minutes followed by 24-hour reperfusion. Humanin G (HNG) (2 mg/kg, iv) was administered 10 minutes before reperfusion. Urine was collected during reperfusion, and the experiment was terminated by collecting blood and tissue samples.

Blood urea nitrogen and serum creatinine levels were elevated in the I/R group and were not affected by HNG treatment. Glutathione levels as well as superoxide dismutase activities, which were diminished in the I/R group, were significantly restored following HNG administration.

Myeloperoxidase activity and malondialdehyde levels were significantly decreased in HN-I/R group compared to the I/R group. ATP levels and mitochondrial Complex I activity were significantly increased in the HN-I/R group compared to I/R. The percentage of apoptotic cells, markedly increased in I/R, was significantly reduced in HN-I/R. STAT3 and ERK 1/2 phosphorylation also increased in HN-I/R rats compared to I/R animals.

HNG exerts a protective effect against renal I/R injury by attenuating oxidative stress, inflammation, and apoptosis while enhancing antioxidant capacity and mitochondrial function, through STAT3 and/or ERK 1/2 activation.

Introduction

Renal ischemia-reperfusion (I/R) injury, which may occur in conditions such as hemorrhage, shock, sepsis, or transplantation, can lead to acute kidney failure, which is associated with high morbidity and mortality rates [9, 52, 51, 36]. I/R injury is a process that begins during the ischemic period and continues to increase during reperfusion [17]. Renal ischemia damages tubular endothelial and epithelial cells, and, if severe, can result in apoptosis and necrosis [74, 77, 4, 50, 54, 27]. During reperfusion, oxygen is resupplied, but this also causes increased formation of reactive oxygen species (ROS) [31, 43]. Increased ROS production exacerbates tissue damage through leukocyte activation and migration, lipid peroxidation of membrane lipids, and oxidative damage to intracellular structures, thereby worsening the degree of apoptosis and necrosis [64, 42, 32, 41].

Mitochondria play a crucial role in ATP synthesis, energy metabolism, regulation of apoptosis, and Ca^{2+} homeostasis [5]. Mitochondrial dysfunction observed in renal I/R injury is characterized by mitochondrial swelling, expansion of cristae and intermembrane spaces, and a reduction in mitochondrial membrane

potential [62, 75, 61]. Damaged mitochondria during I/R inhibit the activity of the electron transport chain (ETC) enzymes, including complex I, complex II, and ATP synthase, resulting in significant electron leakage, hence the increased ROS production [48, 60]. It is well known that the balance between the oxidant and antioxidant systems is maintained under physiological conditions, and this balance is disrupted in I/R injury due to both increased ROS production and a weakened antioxidant system [31, 49]. Various antioxidant agents, such as catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH), have been shown to mitigate ROS-mediated injury during renal I/R. Evidence from experimental kidney I/R models indicates that oxidative stress-related tissue damage is strongly linked to diminished GSH levels. [4, 58, 55, 39].

HN is a mitochondria-derived peptide identified first in amyloid-beta plaques of Alzheimer's patients [63, 25, 24]. HN is known to improve mitochondrial functions, enhance the antioxidant system, and have an anti-apoptotic effect [59, 12, 21]. It has been shown that HN exhibits protective and therapeutic effects against I/R injury in cerebral, myocardial, and intestinal tissues [1, 38, 81, 68]. Additionally, it has been demonstrated that HN exerts its cytoprotective and anti-apoptotic effects under cellular stress conditions in various cell types [23, 82].

Although the intracellular signaling mechanisms underlying the effects of HN have not yet been fully elucidated, current evidence highlights the signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK 1/2) pathways as key mediators of its cytoprotective and anti-apoptotic effects [18, 34, 26]. Various preconditioning and postconditioning interventions that attenuate I/R injury have been shown to activate both STAT3 and ERK1/2 signaling pathways. During reperfusion, levels of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) rise rapidly, triggering downstream signaling cascades involving pathways such as JAK/STAT3 and ERK1/2 [78, 72]. ERK1/2, a member of the MAPK pathway, is involved in many cellular processes including cell proliferation, survival, differentiation, and apoptosis, can be activated by ROS and pro-inflammatory mediators, especially during reperfusion [79]. Although overstimulation of ERK1/2 signaling can contribute to inflammation, a certain level of ERK1/2 activation is crucial for the induction of cell survival, proliferation, and repair signaling during I/R injury, as it is known to be involved in reperfusion injury salvage kinase (RISK) signaling [79, 78]. On the other hand, STAT3 activation in intrinsic protective pathways (such as the SAFE pathway) has been associated with improved mitochondrial function and reduced infarct size in cardiac I/R injury models [28]. This coordinated activation appears to represent an adaptive compensatory response that limits apoptosis, preserves mitochondrial function, and restrains excessive inflammatory signaling [15, 78].

A potent humanin analogue, S14G-humanin (HNG), is generated by replacement of Ser14 with glycine, and this substitution potentially increases humanin activity [25, 24]. The present study used HNG to evaluate its potential protective or therapeutic effects against renal I/R injury in vivo and to identify the potential mechanism/s underlying this protection. Although there are few studies examining the effects of HNG in experimental chronic renal failure models, its acute effects on renal I/R injury, which is accepted as an experimental model of acute kidney injury (AKI) has not yet been investigated.

In the present study, we aimed to investigate the effects of acute HNG administration and the underlying mechanisms in a rat model of AKI induced by renal I/R.

Material and Methods

Animals and Grouping

Forty-eight male Sprague-Dawley rats aged 8–10 week, weighing 200–250 g were used in this study. The animals were housed in stainless steel cages with ad libitum access to food and water. They were maintained under a 12-hour light/dark cycle in a controlled environment with a temperature of $22 \pm 2^{\circ}\text{C}$ and relative humidity ranging from 60% to 70%. All experimental procedures involving rats were conducted in accordance with the guidelines established by the Animal Care and Usage Committee of Akdeniz University (Approval No: 2020.08.004-2024.04.007).

Rats were randomly divided into four groups: Sham (n = 12), Ischemia/Reperfusion (I/R; n = 12), Humanin-Sham (HN-Sham; n = 12), and HN-I/R (n = 12). The Sham groups did not undergo I/R procedures, whereas the IR groups were subjected to 45 minutes of bilateral renal ischemia followed by a 24-hour reperfusion period.

Induction of Renal I/R Injury

All rats were anesthetized with intraperitoneal (ip) ketamine/xylazine (90/10 mg/kg). The tail vein of each rat was cannulated with a 24-G cannula for injections. In the sham groups, a midline abdominal incision was made to expose the renal artery and vein without applying the I/R protocol [77].

In the I/R groups, renal blood flow was interrupted by clamping the renal pedicle with a clamp for 45 min to establish the renal I/R injury model. Upon completion of the ischemic period, the clamps were removed to initiate reperfusion, and the animals were subjected to a 24-hour reperfusion period. In the HN-Sham and HN-I/R groups, the HN analog (HNG) was administered intravenously via the tail vein at a dose of 2 mg/kg, 10 minutes before the reperfusion period. During the reperfusion period, the rats were housed in metabolic cages, and urine samples were collected over 24 hours [77]. At the end of reperfusion, the animals were euthanized, blood samples and kidney tissues were collected for further analysis. HNG; [S14G]-humanin (amino acid sequence: Met-Ala-Pro-Arg-Gly-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Gly-Glu-Ile-Asp-Leu-Pro-Val-Lys-Arg-Ala) was purchased from GenScript Biotech Corporation and used as a HN analog.

Assessment of Renal Function

Blood samples (0.5 ml) were collected and centrifuged to obtain supernatants. Serum creatinine (sCr) and blood urea nitrogen (BUN) levels were assessed using an autoanalyzer (Brand: ROCHE COBAS,

Model: C502) at the Central Laboratory of Akdeniz University Hospital. Creatinine clearance (ClCr) as an indicator of glomerular filtration rate (GFR) was calculated using the following formula [71].

$$\text{ClCr (ml/min)} = \text{Urine (u) Cr (mg/dl)} \times \text{urine flow (ml/min)} / \text{serum (s) Cr (mg/dl)}$$

Histopathological Assessment

The formalin-fixed and paraffin-embedded kidney was cut into 5 µm sections and stained with Haematoxylin and Eosin (H&E) for histological assessment. Histological injury scores were determined by assessing tubular necrosis, loss of the brush border, tubular cast formation, and tubular dilation as described previously [13, 70]. The histological damage scores were counted in a blind study by two observers.

Apoptosis Assessment

Apoptotic cells in rat kidney tissue were evaluated using a Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay Kit (Abcam, Catalog No: ab206386) in accordance with the manufacturer's instructions. TUNEL-stained sections were evaluated using a light microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Five randomly selected slides, each with four different fields at 10x10 magnification, were evaluated for the analysis of TUNEL. Positively stained cell numbers in the fields were counted in a blind study by two observers. Mean positive cell numbers were calculated and then were rated to 100. Positively stained apoptotic cell numbers (apoptotic index) in the tissue were indicated as percent (%).

Western Blot Analysis

The protein concentration of each sample was determined using a BCA assay kit. Equal amounts of protein (20 µg/ml) were loaded into each well and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and 30 mA for 2 hours. The separated proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using a transfer system (Trans-Blot Turbo, Bio-Rad) at 25 V and 1.3 A for 10 minutes [20].

The membranes were incubated overnight with primary antibodies for p-STAT3 (Cat. No: 9145T, Cell Signaling) at 1:1000 dilution, p-ERK1/2 (Cat. No: 4370T, Cell Signaling) at 1:1000 dilution, and beta-actin. A Goat anti-rabbit IgG conjugated secondary antibody was used in accordance with the primary antibodies. The membranes were incubated with a chemiluminescent substrate for 5 minutes, and the bands were visualized using an Azure C280 imaging system. Proteins on the p-STAT3 and p-ERK1/2 membranes were then incubated overnight with the beta-actin antibody (4970, Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:5000, served as an internal control. The obtained results were analyzed using Image J software (Image J Version 1st36b; National Institutes of Health, MD, USA) and protein expression was quantified by normalizing the target protein to the reference protein.

Assessments of Mitochondrial Functions

Intracellular ATP Levels

ATP levels in tissue homogenates were quantified using an ATP Assay Kit (Elabscience, Catalog No: E-BC-K157-S) following the manufacturer's instructions and expressed as ATP content per gram of tissue.

Complex I Activities

Complex I activity in kidney tissue homogenates was determined using a Complex I Activity Assay Kit (Abcam, Catalog No: ab109721), following the manufacturer's protocol. Results were expressed as enzyme activity per milligram of tissue.

Assessments of Oxidative Stress

Malondialdehyde (MDA) levels, which are indicators of lipid peroxidation, were determined in kidney tissue homogenate by the reaction of MDA with thiobarbituric acid (TBA) (Sigma-Aldrich, T5500) and detected using spectrophotometric methods as described previously [11]. MDA levels were expressed as nmol MDA/g tissue.

Assessments of Antioxidant Status

GSH levels, SOD and CAT activity were examined as antioxidant parameters in kidney tissue homogenates.

The level of endogenous antioxidant GSH in tissue was measured by modifying the Ellman method, which detects sulfhydryl groups [16, 3]. Absorbances were measured on a Shimadzu UV-1601 spectrophotometer (at 412 nm), and the calculated GSH level was expressed as $\mu\text{mol/g}$ tissue.

SOD activity was determined using a previously described method [44]. The superoxide radical generated by riboflavin under fluorescent light (20 W) is converted to hydrogen peroxide (H_2O_2) by SOD and subsequently H_2O_2 is detected using o-dianisidine hydrochloride. SOD activity was calculated from the difference between reaction absorbance and basal absorbance at 460 nm in kidney tissue and was expressed as U/g tissue [47].

CAT activity in kidney tissue homogenates was determined using spectrophotometric methods as previously described by Aebi [2]. Absorbance was measured at 240 nm using a spectrophotometer (Shimadzu UV-1601). CAT activity was calculated based on an extinction coefficient of $0.004 \text{ mM}^{-1} \cdot \text{mm}^{-1}$ and expressed as units per gram of tissue (U/g tissue).

Assessments of Inflammation

Kidney tissue Myeloperoxidase (MPO) activity was evaluated as an indicator of inflammation. MPO activity was determined through the reaction of H_2O_2 oxidation with o-dianisidine HCl [10]. Absorbance

changes due to the reaction were measured at 460 nm using a spectrophotometer (Shimadzu UV-1601). MPO activity was expressed as units per gram of tissue (U/g tissue).

Statistical Analysis

Data are presented as mean $\pm$ S.E.M. Significant differences were determined by one-way ANOVA followed by Tukey's post hoc test, and Kruskal–Wallis test followed by Dunn's multiple comparison post hoc test using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Values of < 0.05 were considered statistically significant.

Results

Effects of HNG Treatment on Renal Functions

As shown in Table 1, rats in I/R groups displayed significant deterioration of renal function 24 h after reperfusion, as reflected by increased levels of sCr and BUN with decreased ClCr (an indicator of GFR) compared to those in sham groups.

Table 1 Effects of HNG Treatment on Renal Functions

Groups Parameters	Sham	I/R	HN-Sham	HN-I/R
sCr (mg/dl)	0,340 $\pm$ 0,02	1,549 $\pm$ 0,34***	0,357 $\pm$ 0,03	1,227 $\pm$ 0,22**
BUN (mg/dl)	18,83 $\pm$ 0,49	75,11 $\pm$ 10,5***	19,00 $\pm$ 0,82	73,11 $\pm$ 12,5***
ClCr(ml/min)	1,06 $\pm$ 0,09	0,38 $\pm$ 0,08***	0,93 $\pm$ 0,1	0,56 $\pm$ 0,18

Data are presented as mean $\pm$ S.E.M. ***p < 0.001 vs. Sham; **p < 0.01 vs. Sham. I/R, ischemia/reperfusion; HN, humanin; ClCr, creatinine clearance; sCr, serum creatinine; BUN, blood urea nitrogen.

Effects of HNG Treatment on Histopathological Damage

Renal tissues were examined after H&E staining (Figure 1a). Structural changes in the tubules, such as tubular necrosis, atrophy, dilation in the tubules and Bowman's capsule, loss of the brush border, and cast formation, were observed and histopathologically scored (Figure 1b). The histopathological damage score in the I/R group was significantly higher compared to the Sham group (p <0.001). The histopathological damage score in the HN-I/R group was also significantly higher compared to the HN-

Sham group ($p < 0.01$). The damage observed in the HN-I/R group was not significantly different from that in the I/R group.

Effects of HNG Treatment on Apoptosis

Apoptotic cells in renal tissues were detected immunohistochemically using the TUNEL kit (Figure 1b). Apoptotic cells were counted, and the percentage of apoptotic cells was calculated (Figure 1d). The percentage of apoptotic cells in the I/R group was significantly higher compared to the Sham group ($p < 0.001$). The apoptotic cell density in the HN-I/R group was significantly lower compared to the I/R group ($p < 0.01$).

Effects of HNG Treatment on Mitochondrial Functions

To evaluate mitochondrial functions, intracellular ATP levels in renal tissue homogenates were measured (Figure 2a). The ATP levels in the I/R group were found to be significantly lower compared to the Sham group ($p < 0.001$). Tissue ATP levels measured in the HN-I/R group were significantly elevated compared to the I/R group ($p < 0.05$).

As an indicator of mitochondrial function, Complex I activity levels were measured in renal tissue homogenates (Figure 2b). The Complex I activity in the I/R group was significantly lower compared to the Sham group ($p < 0.01$). However, the Complex I activity levels in the HN-I/R group were significantly higher compared to the I/R group ($p < 0.05$).

Effects of HNG Treatment on Oxidative Stress and Antioxidant Status

Levels of MDA, which is the product of lipid peroxidation in renal tissue homogenates, are presented in Figure 3a. MDA levels were significantly higher in the I/R group compared to the Sham group ($p < 0.001$). In the HN-I/R group, MDA levels were not significantly different from those in the HN-Sham group. However, tissue MDA levels were significantly lower in the I/R group treated with HNG compared to the untreated group ($p < 0.05$).

Renal tissue GSH levels as shown in Figure 3b were significantly reduced in the I/R group compared to the Sham group ($p < 0.001$). In the HN-I/R group, GSH levels did not differ significantly from those in the HN-Sham group. However, renal GSH levels in the HN-I/R group were significantly higher than those observed in the I/R group. ($p < 0.01$).

SOD activity, the most effective endogenous antioxidant enzyme in cellular defense, was measured as an indicator of antioxidant capacity in renal tissue homogenates (Figure 3c). SOD activity in the I/R group was significantly lower compared to the Sham group ($p < 0.05$). In the HN-I/R group, SOD activity was not significantly different from that in the HN-Sham group. However, renal SOD activity in the HN-I/R group treated with HNG prior to reperfusion was significantly higher compared to the I/R group ($p < 0.05$).

CAT activity was measured as an antioxidant marker in renal tissue homogenates (Figure 3d). CAT activity in the I/R group was significantly lower compared to the Sham group ($p < 0.001$). Similarly, CAT activity in the HN-I/R group was significantly lower compared to the HN-Sham group ($p < 0.001$). No significant difference was found in CAT activity between the HN-I/R group and the I/R group.

Effects of HNG Treatment on Inflammation

MPO, as a marker of neutrophil infiltration into the damaged tissue, was assessed to evaluate the inflammatory response, was significantly elevated in the I/R group compared to the Sham group ($p < 0.001$) (Figure 3e). Similarly, MPO activity in the HN-I/R group was significantly higher compared to the HN-Sham group ($p < 0.001$). Moreover, MPO activity in the HN-I/R group was significantly reduced compared to the I/R group, indicating a potential attenuation of inflammatory response ($p < 0.05$).

Effects of HNG Treatment on ERK1/2 and STAT3 Phosphorylation

Western blot analyses demonstrated significant alterations in ERK1/2 and STAT3 signaling among the experimental groups (Figure 4 a,c). ERK1/2 phosphorylation was increased in the I/R group compared to the Sham group; however, this difference was not statistically significant. In contrast, Phosphorylated ERK 1/2 (pERK1/2) levels were significantly higher in the HN-I/R group compared with both the HN-Sham and I/R groups ($p < 0.001$).

Similarly, Phosphorylated STAT3 (pSTAT3) expression was significantly increased in the I/R group compared to the Sham group ($p < 0.01$). pSTAT3 levels in the HN-I/R group were markedly elevated compared with both the I/R and HN-Sham groups ($p < 0.001$) (Figure 4b,d). Beta-actin expression did not differ among groups, confirming equal protein loading. These results indicate that HNG treatment is associated with increased activation of ERK1/2 and STAT3 signaling pathways in renal tissue following I/R injury.

Discussion

Renal I/R injury, frequently arises from kidney transplantation hemorrhage and nephrectomy, remains a significant unresolved challenge in clinical practice. Renal I/R injury is one of the main causes of AKI which leads to disease with high mortality and morbidity [4,9]. During I/R injury, tissue damage initiated during the ischemic phase persists and further exacerbates upon reperfusion [56,32]. The main mechanism of renal IR injury is associated with multiple mediators including oxidative stress, inflammation, and mitochondrial dysfunction activation of adhesion molecules, ultimately resulting in renal tubular damage and apoptosis [69,8,61,75]. At present, strategies targeting apoptosis and oxidative stress may contribute to maintaining renal function and reducing tubular injury [27,22].

In the present study, we aimed to investigate the effects of HN on renal I/R injury and to elucidate the potential mechanisms involved, using HNG as an HN analog. To the best of our knowledge, this is the first study to examine the effects of HNG administration on renal I/R injury. We found that HNG

administration attenuated the pathological alteration in renal I/R injury, which was accompanied with reduced inflammation, oxidative stress and apoptosis. Additionally, HNG preserved mitochondrial functions, increased Complex I activity and elevated ATP levels. These findings demonstrate that HNG confers protective effects against renal I/R injury in rats. Furthermore, our data suggest that HNG-mediated renoprotection may involve STAT3 and ERK 1/2 signaling pathways.

In I/R-induced AKI, the decline in GFR is accompanied by an increase in circulating nitrogenous waste products, ultimately resulting in impaired renal function [53,43]. Although our findings confirmed the development of renal dysfunction following I/R injury, they also indicated that HNG treatment did not produce a significant improvement in reduced GFR or in the elevated sCr and BUN levels, which represent key markers of impaired renal function. This outcome may be attributed to several factors, including the administration of HNG immediately before reperfusion rather than during the ischemic phase, its use as a single dose, and the absence of repeated dosing.

In our study, renal tissue injury was assessed through histopathological examination and apoptosis. It is well known that necrosis is the primary pathway of cell death during ischemia in renal I/R injury [36,73]. The tubular necrosis parameters, which were elevated in the I/R group, did not show a significant reduction following HNG treatment in our study. Several previous studies conducted in different tissues have demonstrated that administration of HNG either prior to or during ischemia leads to a reduction in necrosis parameters [68]. We believe that the most plausible explanation for the discrepancy between our findings and those reported in the literature is the timing of HNG administration. In our study, HNG was administered 10 minutes prior to reperfusion to specifically investigate its effects on reperfusion-induced injury [46]. In contrast, the aforementioned studies utilized HNG as a preconditioning agent administered before the onset of ischemia, thereby focusing on its protective role against ischemia-induced necrosis [68,1].

Accumulating evidence has suggested that apoptosis is critically involved in the pathological process of renal I/R injury [75,27,54]. The present study also demonstrated that I/R caused a significant increase in TUNEL-positive tubular cells, indicating the occurrence of apoptosis. Moreover, the findings of this present study revealed that HNG treatment prior to reperfusion significantly reduced the apoptosis in renal tubular cells. Anti-apoptotic effects of HN have been demonstrated in numerous *in vitro* and *in vivo* studies, including I/R injury models [21,29,66,1]. Previous investigation has indicated that HN inhibits apoptosis by increasing the expression of the anti-apoptotic protein Bcl-2 and decreasing the expression of the pro-apoptotic proteins Bax and suppression of caspase-3 activation in I/R injury [21,29,1]. pSTAT3 has been demonstrated to play a direct anti-apoptotic role in I/R injury by increasing the expression of the anti-apoptotic protein Bcl-2 and decreasing the expression of the pro-apoptotic proteins Bax and suppression of caspase-3 activation [80]. In light of these findings, it could be argued that anti-apoptotic properties of HNG is dependant on STAT3 and/or ERK 1/2 phosphorylation , consistent with our results.

Disruption of cellular energy homeostasis, particularly due to compromised mitochondrial activity, represents a central mechanism in the pathogenesis of renal I/R injury [61,5]. It is well established that

renal I/R injury induces mitochondrial dysfunction, including a reduction in ETC enzymes activity and ATP production [30,40]. This phenomenon plays a crucial role in I/R induced damage in tubular epithelial cells, which are characterized by increased mitochondrial density. Previous studies have suggested that HN increases intracellular ATP levels in various cell types including neurons, muscle cells, and pancreatic β -cells consistent with our findings [33,37,14,76]. The results of the present study also demonstrated that HN treatment restored intracellular ATP levels and Complex I activity, both of which were reduced by I/R injury. pSTAT3 has been shown to enhance ATP production in hepatic I/R injury by increasing the activity of mitochondrial respiratory chain complexes I and II [78]. In our study, the observed increase in ATP levels and complex I activity following HNG treatment may have been mediated by pSTAT3-dependent mechanisms.

Increased production of ROS, which are primarily generated due to mitochondrial dysfunction during renal I/R injury significantly contribute to tissue injury [5,48,30]. MDA, as an end product of lipid peroxidation induced by ROS, can directly cause cytotoxic damage [11,19]. On the other hand, antioxidants such as SOD, CAT and GSH possess protective effects against oxidative stress [6]. It was accepted that the imbalance between oxidants and anti-oxidants can reflect the level of ROS metabolism and tissue damage [58]. In the present study, we observed a significant increase in renal MDA and decrease in renal GSH level and SOD activity in I/R rats, while HNG administration down-regulated MDA and upregulated SOD activity and GSH levels. Similarly to our findings, previous studies have suggested that HN suppresses oxidative stress while enhancing the antioxidant defense system in I/R models involving various tissues including cardiac and cerebral tissues as well as in diverse cell types [81,35]. One of the proposed mechanisms underlying the anti-oxidative effects of HNG is its ability to directly modulate mitochondrial function [38]. It is proposed that HNG exerts this effect either through mitochondrial colocalization and/or via activation of the STAT3 signaling pathway [18,67] that is consistent with our results (Figure 4b). Moreover, pSTAT3 has also been reported to inhibit the formation of the mitochondrial permeability transition pore (mPTP), thereby preserving the mitochondrial membrane potential ($\Delta\Psi_m$), reducing the generation of ROS, and suppressing apoptosis [7].

In the present study HNG treatment did not improve CAT activity, which was reduced by I/R injury. During renal I/R, unlike other antioxidant enzymes, both CAT activity and CAT protein synthesis are known to decrease [58]. The inability of HNG to enhance CAT activity observed in our study may be attributed to a reduction in CAT protein levels. Indeed, previous studies employing in experimental I/R models have demonstrated that HN treatment preserves SOD and glutathione peroxidase activities in intestinal and brain tissues following I/R injury. However, CAT activity was not evaluated in those investigations [81,1].

It has been verified that during renal I/R injury, inflammation is one of the most important mechanisms involved in tissue damage [69,56]. On the other hand, HN has been characterized as an anti-inflammatory agent due to its suppressive effects on pro-inflammatory cytokines [81,57]. It has been shown that HN reduces age-related neuroinflammation and suppresses pro-inflammatory cytokines in an intestinal I/R model, in diabetic nephropathy and traumatic brain injury [1,45,65]. In our study, to investigate the inflammatory response associated with renal I/R injury, we assessed MPO activity as a marker of

neutrophil infiltration into kidney tissues. Consistent with previous findings, our results also demonstrated that HNG treatment significantly attenuated I/R-induced inflammation in renal tissue.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that HNG has renoprotective effects against AKI induced by renal I/R injury. According to the findings of our study, HNG exerts renoprotective effects, at least partly, by promoting STAT3 and/or ERK 1/2 activation, thereby enhancing antioxidant, anti-apoptotic, and anti-inflammatory responses, as well as regulating mitochondrial functions. These results suggesting that HNG may be a promising agent in the management of AKI.

Declarations

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Ethics approval and consent to participate All experimental procedures involving rats were conducted in accordance with the guidelines established by the Animal Care and Usage Committee of Akdeniz University (Approval No: 2020.08.004-2024.04.007).

Consent for publication All authors approved the publication of this manuscript.

Competing interests The authors declare no competing interests.

Data availability

The corresponding author is willing to provide the data and datasets used and/or analyzed during the current study upon reasonable request.

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Figures

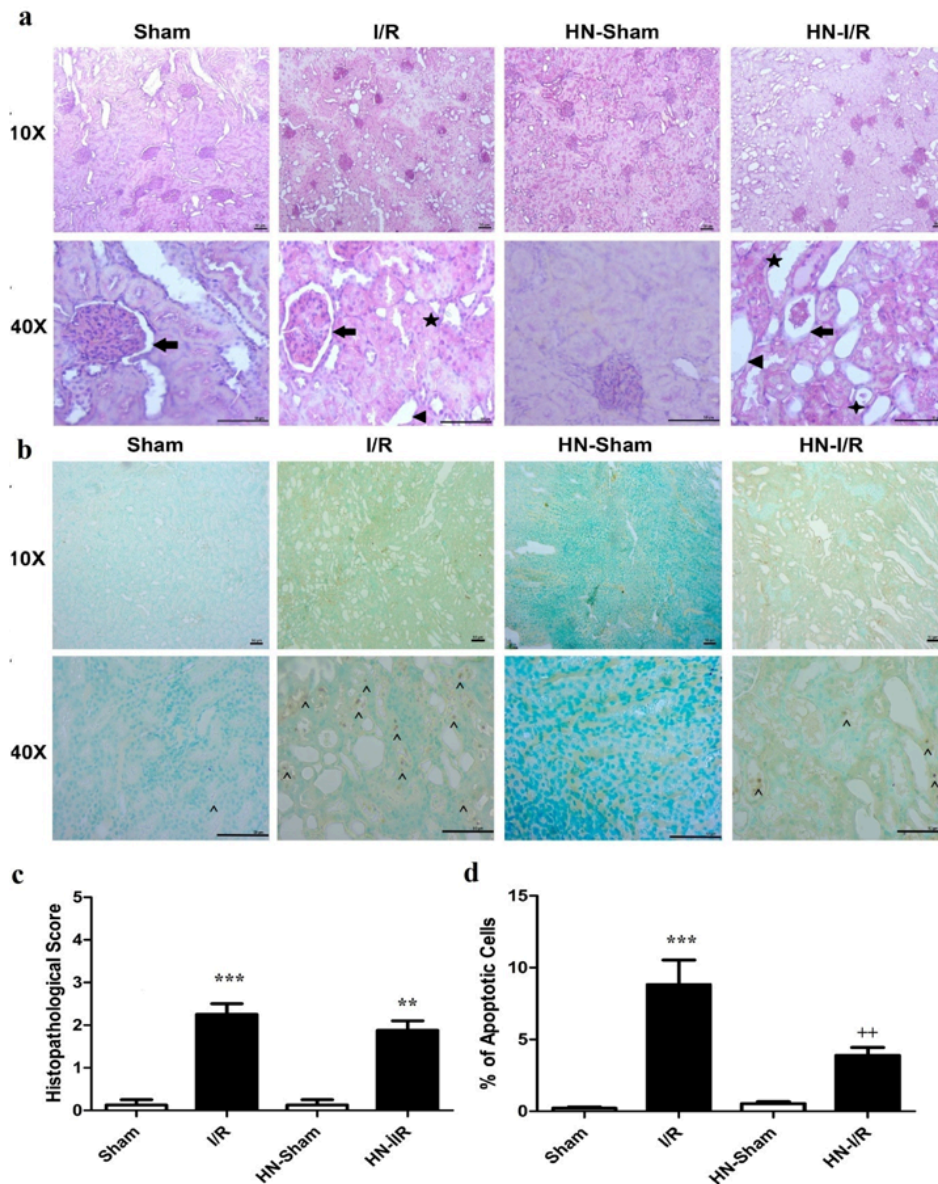


Fig. 1 Histopathological damage and apoptosis in renal tissue after I/R injury and HNG treatment. (a) Representative H&E-stained kidney sections showing tubular necrosis (►), tubular dilation (◄), cast formation (✚), and loss of brush border (★) in experimental groups. (b) Representative TUNEL-stained kidney sections showing apoptotic cells (brown-stained nuclei). (c) Semi-quantitative histopathological injury scores based on tubular necrosis, tubular dilation, cast formation, and brush border loss. (d) Quantification of apoptotic index (% TUNEL-positive cells). Data are presented as mean ± S.E.M. *** $p < 0.001$ vs. Sham; ** $p < 0.01$ vs. I/R. I/R, ischemia/reperfusion; HN, humanin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Figure 1

See image above for figure legend.

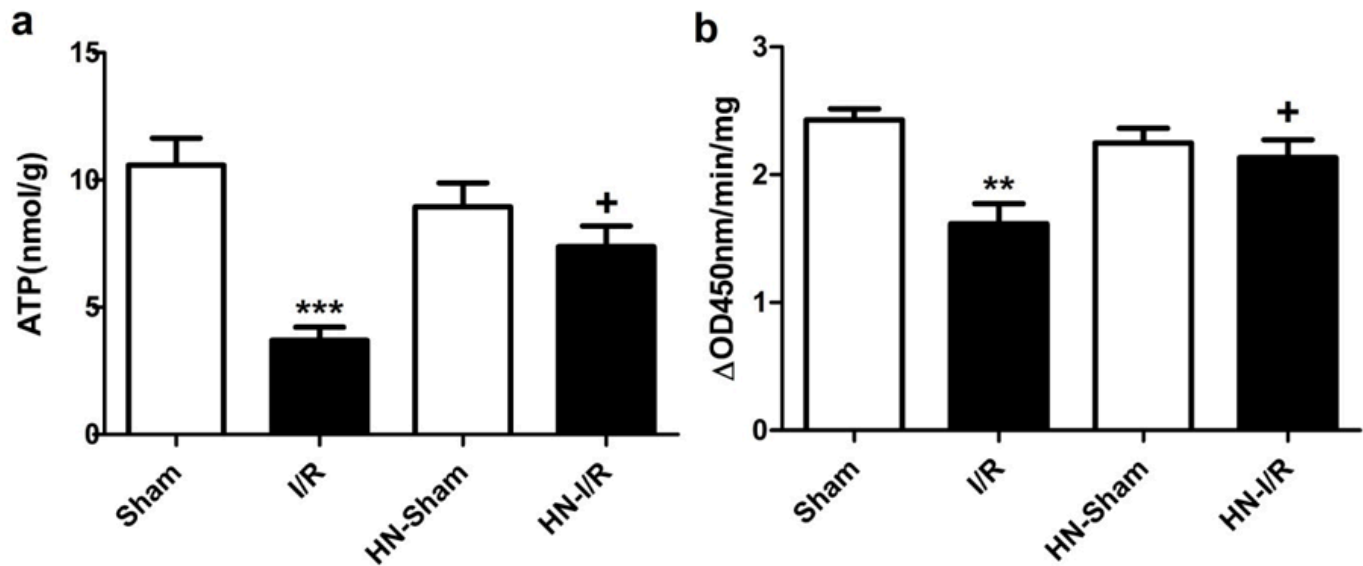


Figure 2

Effects of HNG on mitochondrial function in renal tissue following I/R injury. (a) Intracellular ATP levels in renal tissue homogenates. (b) Mitochondrial Complex I activity in renal tissue homogenates. Data are presented as mean $\pm$ S.E.M. *** $p < 0.001$ vs. Sham; ** $p < 0.01$ vs. Sham; * $p < 0.05$ vs. I/R. I/R, ischemia/reperfusion; HN, humanin; ATP, adenosine triphosphate

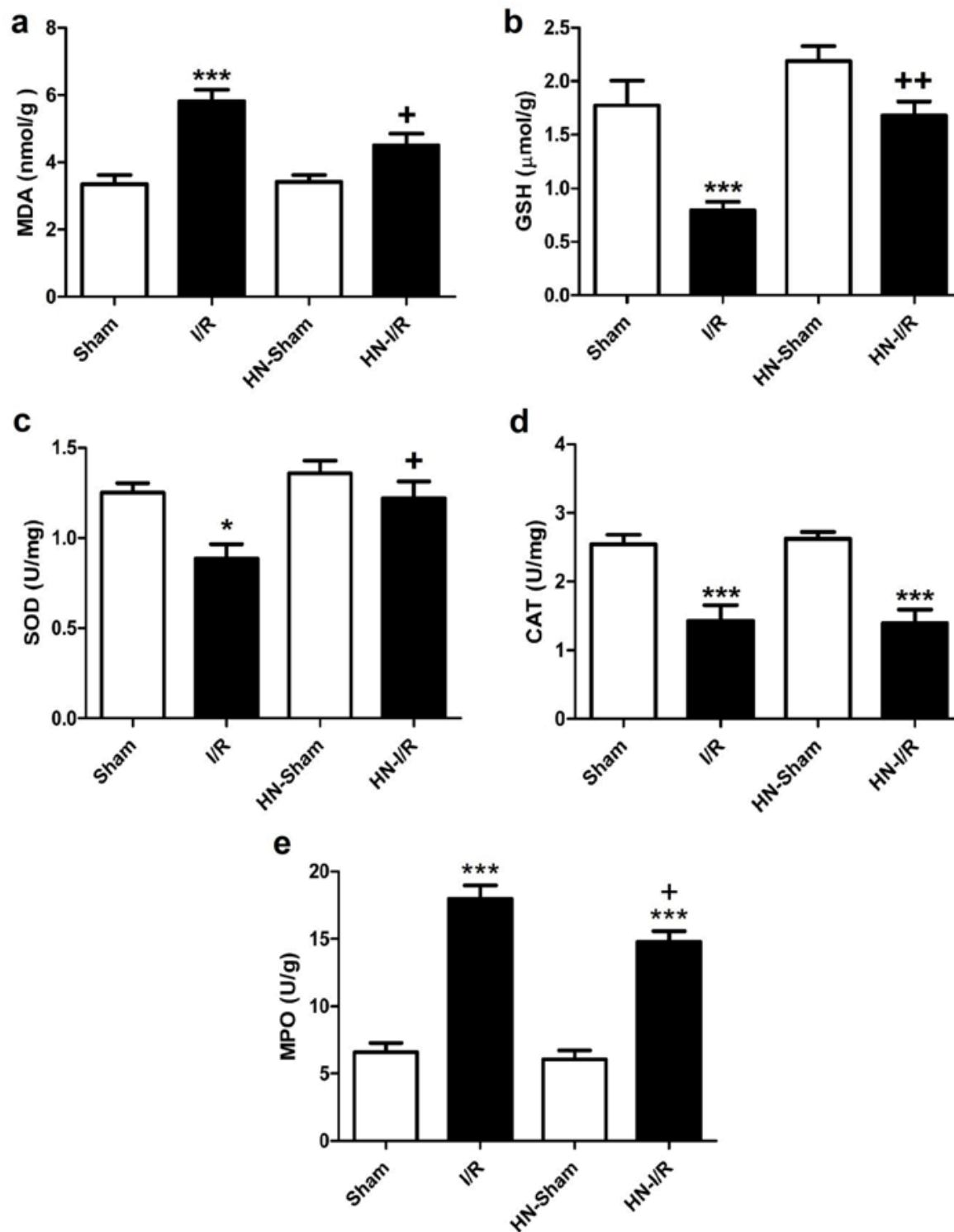


Figure 3

Effects of HN on oxidative stress, antioxidant status, and inflammation in renal tissue. (a) Malondialdehyde (MDA) levels. (b) Glutathione (GSH) levels. (c) Superoxide dismutase (SOD) activity. (d) Catalase (CAT) activity. (e) Myeloperoxidase (MPO) activity. Data are presented as mean $\pm$ S.E.M. *** p < 0.001 vs. Sham; ** p < 0.01 vs. Sham; * p < 0.05 vs. Sham; ++ p < 0.01 vs. I/R; + p < 0.05 vs. I/R. I/R,

ischemia/reperfusion; HN, humanin; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; MPO, myeloperoxidase

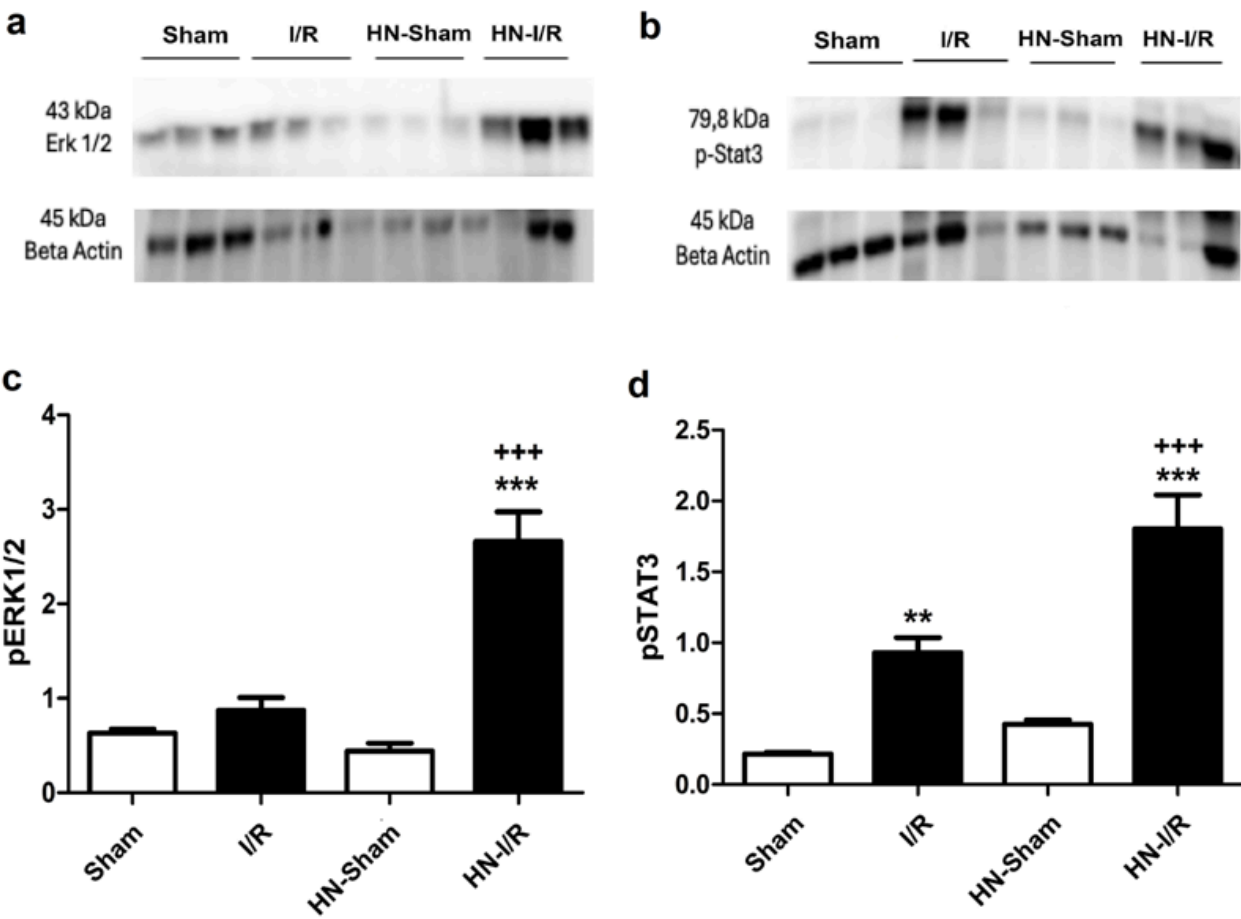


Figure 4

Effects of Humanin G Treatment on p-ERK1/2 and p-STAT3 phosphorylation. Following Ischemia-Reperfusion (IR) Injury. (a,b) Representative Western blot bands of p-ERK1/2 and p-STAT3 protein levels in SHAM, IR, HN SHAM, and HN IR groups. Beta Actin (45 kDa) was used as a loading control to ensure equal protein distribution. (c,d) Densitometric analysis of the immunoreactive bands expressed as relative protein levels. Data are presented as mean $\pm$ SEM. **p < 0.01, **p < 0.001 vs. SHAM group; +++p < 0.001 vs. IR group