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ORIGINAL ARTICLE

Protective Mechanisms of Piperine against Renal Ischemia–Reperfusion Injury in Rats

Mohamed Aly Morsy[1,2], Mahmoud El-Daly[3], Anwaar S. M. Abu Shnaf[4], Sherif W. Mansour[5], Ahmed R. N. Ibrahim[6,7]

[1]Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia, [2]Department of Pharmacology, Faculty of Medicine, Minia University, [3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, [4]Department of Zoology, Faculty of Science, Minia University, El-Minia, [5]Department of Physiology, Faculty of Medicine, Zagazig University, Zagazig, Egypt, [6]Department of Clinical Pharmacy, College of Pharmacy, King Khalid University, Abha, Saudi Arabia, [7]Department of Biochemistry, Faculty of Pharmacy, Minia University, El-Minia, Egypt

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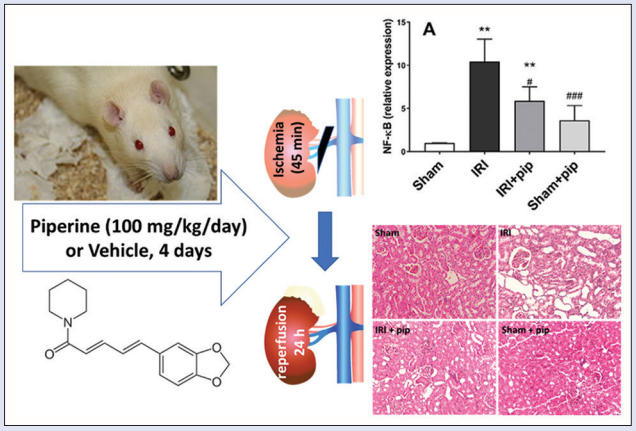
ABSTRACT

Background: Renal ischemia–reperfusion injury (IRI) is a major clinical problem associated with kidney transplantation, leading to high mortality and morbidity. IRI involves activation of oxidative stress and inflammatory pathways, eventually leading to cell death and organ failure. Piperine is a phenolic active ingredient of black pepper, which showed promising antioxidant and anti-inflammatory potential. **Objectives:** We hypothesized that piperine would protect against renal IRI in rats via inhibition of oxidative stress and inflammation. **Materials and Methods:** Male Sprague Dawley rats were subdivided into four groups; sham, IR, IR + piperine, and sham + piperine. All animals have been treated for 4 days with either vehicle or piperine (100 mg/kg/day). One hour after the last piperine or vehicle administration, animals were subjected to bilateral renal ischemia for 45 min by clamping both renal pedicles, followed by reperfusion for 24 h. At the end of the experiments, kidneys were harvested for the determination of lipid peroxidation (malondialdehyde [MDA]), reduced glutathione (GSH), inflammatory and apoptotic markers, and histopathology. Serum levels of creatinine and urea have been determined. **Results:** Induction of renal IR increased renal oxidative stress (increased MDA and decreased GSH) and the expression levels of inflammatory and proapoptotic genes (nuclear factor-kappa B, inducible nitric oxide synthase, cyclooxygenase-2, and caspase-3). Moreover, serum levels of creatinine and urea were significantly elevated. Alternatively, pretreatment of the animals with piperine resulted in normalization of these parameters. **Conclusion:** The results showed that piperine pretreatment protects against IRI in rat kidneys via mechanisms involving amelioration of oxidative stress along with inflammatory and apoptotic pathways.

Key words: Apoptosis, inflammation, oxidative stress, piperine, renal ischemia–reperfusion

SUMMARY

- Induction of renal ischemia–reperfusion increased renal oxidative stress and the expression levels of inflammatory genes
- Piperine pretreatment attenuated renal tissue injury, levels of oxidative stress and expression of inflammatory markers
- Piperine protection is due to its antioxidant and anti-inflammatory effects.



Abbreviations used: COX-2: Cyclooxygenase-2; GSH: Reduced glutathione; H&E: Hematoxylin and eosin; iNOS: Inducible nitric oxide synthase; IRI: Ischemia–reperfusion injury; MDA: Malondialdehyde; NF-κB: Nuclear factor-kappa B; PCR: Polymerase chain reaction; TNF-α: Tumor necrosis factor-alpha.

Correspondence:

Prof. Mohamed Aly Morsy,
Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa 31982, Saudi Arabia.
E-mail: momorsy@kfu.edu.sa

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INTRODUCTION

Renal ischemia–reperfusion injury (IRI) is a major cause of acute kidney disease that occurs during renal transplantation, resulting in high rates of morbidity and mortality. Renal transplantation patients are at high risk of early graft rejection and decreased long-term survival due to IRI of the transplanted kidney. Thus, IRI is the main cause of early graft rejection and has a long-term effect on the survival of the transplanted kidney.[1,2] In addition, patients at high risk of cardiovascular events, including but not restricted to sepsis, shock, surgery, and infections,[3,4] are also prone to renal IRI and acute kidney injury as a usual subsequence.[5,6]

Multiple mechanisms underlie the pathological changes during IRI, among which activation of inflammatory pathways represents the cornerstone.[5-7] As a result of IRI, neutrophils are activated and tissue infiltration increases.[8] This mechanism is usually associated with increased production of inflammatory cytokines,[9,10] adhesion molecules,[11,12] as well as increased production of reactive oxygen species,[3,13] all of which contribute to renal cell death and eventually organ failure.[14,15]

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Piperine is a phenolic principle that is the main active ingredient of black pepper (*Piper nigrum* L. of the family Piperaceae) and is responsible for its characteristic pungent taste.[16] Accumulating evidence showed promising antioxidant and anti-inflammatory effects of piperine, which made it a potential candidate for addressing various inflammatory conditions.[17-19] The pharmacological effects attributed to this alkaloid include being anti-inflammatory, via modulation of immune cell proliferation and maturation, suppression of inflammation-induced nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (COX-2) upregulation,[19] as well as inhibiting lipopolysaccharide-induced inflammatory signaling.[20] Of most relevance to the current study, piperine was found to be protective against cerebral and intestinal IRIs in experimental animal models.[21-23] Moreover, a recent report showed that piperine treatment was protective against renal IRI in Wistar rats.[24]

Despite continued research efforts, the scientific community is yet to find proper treatment for renal IRI. Such treatment would increase the success rate of renal transplantation surgeries and protect the kidney against ischemic insults. Given its established anti-inflammatory and oxidative stress-modulating effects, we hypothesize that pretreatment with piperine can protect against renal IRI in rats, perhaps by amelioration of IRI-mediated inflammatory signaling. The aims of the current study were to establish the possible protective effects of piperine against IRI of the kidney and to determine the possible mechanisms involved in such protection.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (n = 28), 200–250 g body weight, were used in this study. The animals were supplied by the animal care facility of the Faculty of Pharmacy, Beni-Suef University, and kept under proper laboratory conditions during the experiment. Rats were allowed free access to tap water and standard chow diet. All procedures of animal use were carried out according to protocols approved by the Commission on the Ethics of Scientific Research, Faculty of Pharmacy, Minia University, Egypt.

Experimental design

Rats were divided randomly into 4 equal groups, 7 rats each. Two animal groups received only the vehicle once daily: sham (negative control) and IRI (positive control). The other two groups received piperine (100 mg/kg) once daily: the IRI + piperine group and the sham control group (sham + piperine). The dose of piperine (100 mg/kg) was selected based on previous experimental work.[25] All treatments were initiated on a once-daily basis for 4 consecutive days. All groups were operated for induction of IRI, except for the sham and the sham + piperine groups. All rats fasted overnight with free access to drinking water. On the 4th day of treatment, rats were injected with thiopental (60 mg/kg, i.p.) for induction of anesthesia. A midline abdominal incision was made, and the two renal pedicles were exposed with minimal dissection and occluded for 45 min with the help of surgical clamps. After 45 min of ischemia, the surgical clamps were removed to allow blood flow (reperfusion period), and the abdominal incision was sutured by surgical silk. The reperfusion phase lasted for 24 h.[26] On the other hand, all steps of the surgical procedure were carried out on the sham and sham + piperine groups but without clamping their renal pedicles.

After the 24-h reperfusion period, animals were sacrificed by cervical dislocation and exsanguination. Blood samples were collected for estimation of serum creatinine and urea levels. The kidneys were immediately harvested. One kidney tissue was homogenized in ice-cold phosphate-buffered saline (20% w/v). RNA was extracted from the tissue homogenate for determination of the expression levels of NF-κB, inducible nitric oxide synthase (iNOS), COX-2, and caspase-3. Tissue levels of malondialdehyde (MDA) and reduced glutathione (GSH), as markers of renal oxidative stress and antioxidant capacity, respectively, were measured in another portion of the homogenate. The other kidney was fixed in a 10% isotonic formalin solution and used for histopathological examination.

Determination of creatinine and urea in serum

Serum samples were used for the determination of creatinine and urea levels using commercially available kits (Diamond Diagnostics, Egypt).

Determination of renal tissue malondialdehyde and glutathione

Lipid peroxidation in the kidney tissues, as a marker of oxidative stress, was determined in tissue homogenates as thiobarbituric acid-reactive species (also referred to as MDA) according to the method established by Buege and Aust.[27] The ability of GSH to reduce Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB) to form the yellow-colored 2-nitro-5-mercaptobenzoic acid product was the basis for determination of renal levels of GSH.[28]

Real-time polymerase chain reaction gene expression of nuclear factor-kappa B, inducible nitric oxide synthase, cyclooxygenase-2, and caspase-3 in rat kidney tissue

The extraction of total RNA from homogenized tissue samples was carried out using the RNeasy Purification Reagent (Qiagen, Valencia, CA, USA), in accordance with the protocol supplied by the manufacturer. The quantity of total recovered RNA was determined spectrophotometrically at 260 nm. Reverse transcriptase reaction was employed to obtain cDNA from the extracted RNA samples (Cat. no. A3500; Promega, Madison, WI, USA). Briefly, total RNA (5 μg) from each sample was mixed with 1 μl of antisense nonspecific primers (20 pmol) and 0.8 μl of the reverse transcriptase (37°C) for a period of 60 min.

The SYBR® Green method was used to assess the relative abundance of the mRNA species in ABI Prism 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The estimated annealing temperature of the primer sets [Table 1] was 60°C. A 25-μl reaction mixture containing 2x SYBR Green Polymerase Chain Reaction Master Mix (Applied Biosystems), 900 nM of each primer, and 2–3 μl of cDNA samples was used. The reaction conditions were set to 2 min at 50°C (one cycle), 10 min at 95°C (one cycle), and 40 cycles of denaturation at 95°C: annealing/extension each at 60°C/70°C, for 15 and 30 s, respectively, followed by a single cycle of 10 min at 72°C. The resulting data were analyzed using the Sequence Detection Software ver. 1.7 (PE Biosystems, Foster City, CA, USA). The comparative Ct method was used to determine the relative expression of the studied genes.

Histological assessment of the kidney

The histopathological examination of kidney samples was carried out. Briefly, samples were excised from experimental groups and kept in 10% isotonic formalin for 1 day. Fixed tissues were gradually dehydrated in alcohol solutions of increasing concentrations, followed by clearing in xylene. Paraffin-embedded kidney tissues were sectioned (4 μm) and hematoxylin and eosin-stained for histopathological examination by a light microscope.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test using GraphPad Prism software (ver. 7.00 for Windows, GraphPad Software, La

Jolla, CA, USA, www.graphpad.com). Statistically significant differences between groups were considered at $P < 0.05$.

RESULTS

Effect of piperine on renal function and structure after ischemia-reperfusion injury

Induction of renal injury by IR in experimental animals resulted in significant deterioration of renal function manifested as elevated serum levels of creatinine and urea measured 24 h after reperfusion when compared with either sham or sham + piperine-treated animals [Figure 1a and b]. On the other hand, pretreatment of rats with piperine protected against IRI-induced kidney function deterioration, as shown by significantly lowered serum levels of creatinine and urea in these animals. Importantly, piperine treatment alone did not alter these kidney function parameters.

Histological sections of kidney tissue from the four studied groups were examined, as shown in Figure 1c. Sham-operated rat kidney histological

Table 1: Primers of studied genes

	Primer sequence	Gene bank accession number
NF-κB	Forward: CATTGAGGTGTATTTCACGG Reverse: GGCAAGTGGCCATTGTGTTC	NM_199267.2
iNOS	Forward: GACCAGAAACTGTCTCACCTG Reverse: CGAACATCGAACGTCTCACA	NM_012611
COX-2	Forward: CCATGTCAAAACCGTGGTGAATG Reverse: ATGGGAGTTGGGCAGTCATCAG	AF233596.1
Caspase-3	Forward: ACTCTTGTGGGCAAACCCAA Reverse: CTCTCCATGAGCAGTAGCCG	NM_001087756.1
Beta-actin	Forward: TATCCTGGCCTCACTGTCCA Reverse: AACGCAGCTCAGTAACAGTC	NM_031144.3

NF-κB: Nuclear factor-kappa B; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase-2

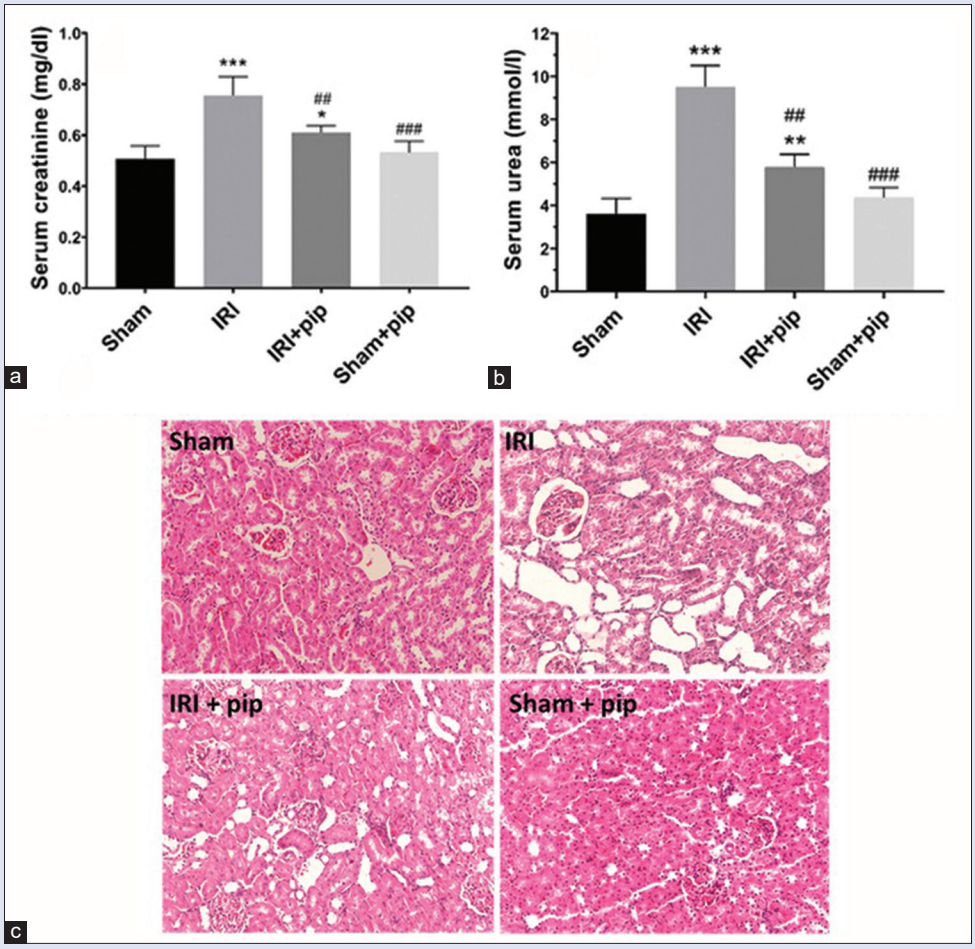

Figure 1: Effect of piperine on serum creatinine (a) and urea (b) and kidney histopathology (c) in renal ischemia–reperfusion injury in rats. Values represent mean ± standard deviation ($n = 7$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the sham group; ##$P < 0.01$, ###$P < 0.001$ versus ischemia–reperfusion injury group. IRI: ischemia-reperfusion injury; Pip: Piperine

sections exhibited normal architecture. The cortex consisted of normal renal corpuscles and intact proximal as well as distal convoluted tubules. The glomeruli are spherical and enclosed by two thin layers of Bowman's capsule. In addition, normal columnar epithelial cells lined the proximal convoluted tubules. Similarly, cuboidal epithelial cells lining the distal convoluted tubules showed normal round and large nuclei. On the other hand, the microscopic examination of the kidney section in the sham + piperine-treated animal group showed some pathological changes. There is a reduction in the lumen of renal tubules and shrinkage in glomeruli. In addition, there is infiltration with some leukocytes. However, greater damage was observed in the kidney sections of the IRI-animal group as compared with the sham and sham + piperine-treated animal groups. There are some necrotic foci in the cortex and some tubular degeneration in epithelial cells. Edema is observed in renal corpuscles (large space between the two thin layers). Furthermore, there is infiltration with some leukocytes. However, in the IRI + piperine animal group, the histopathology of the kidney was improved than in IRI-animals, whereas the necrotic regions were fewer and the glomeruli appeared normal, more or less like those seen in the sham group.

Effect of piperine on renal ischemia–reperfusion injury-induced oxidative stress

Animals exposed to renal ischemia in both kidneys for 45 min followed by reperfusion for 24 h showed significantly elevated levels of MDA, a marker of oxidative stress and lipid peroxidation [Figure 2a] when compared to sham-operated rats. In addition, kidney tissues from these animals showed a parallel decrease in GSH levels [Figure 2b]. Conversely, pretreatment of IRI animals with piperine normalized tissue levels of MDA and partially salvaged renal levels of GSH [Figure 2]. Nevertheless, no significant differences were observed between sham animals that received piperine and sham (control animals) with regard to MDA or GSH levels.

Effect of piperine on renal ischemia–reperfusion injury-induced inflammatory and apoptotic gene expressions

Induction of renal IRI induced a significant elevation (10.43 ± 2.60-fold) in the gene expression of the inflammatory transcription factor NF-κB in comparison to sham animals [Figure 3a]. This effect was significantly attenuated in animals pretreated with piperine before the induction of IRI (5.88 ± 1.63). However, these animals still showed higher than normal levels of NF-κB gene expression. On the other hand, although sham-treated animals that received only piperine showed higher levels of NF-κB, this increase was not significantly different from untreated sham controls.

Similarly, IRI-positive control animals showed increased expression levels of iNOS, COX-2, and caspase-3 messages [Figure 3a-d, respectively], which were parallel to the increase in NF-κB expression [Figure 3a]. On the other hand, piperine-pretreated rats (IRI + piperine) had significantly lower expression levels of these genes. Noteworthy, piperine alone showed some elevations of iNOS, COX-2, and caspase-3 mRNA levels, albeit insignificant when compared to sham untreated animals.

DISCUSSION

The aims of this study were to establish the nephroprotective effects of piperine, an alkaloid of *P. nigrum*, against IR-induced kidney damage in rats and to further understand its mechanism of protection. Our hypothesis was that piperine will protect the kidneys against IRI via its antioxidative and anti-inflammatory mechanisms. The results showed that the induction of renal IRI was manifested as deterioration of renal function parameters (serum creatinine and urea), histopathological abnormalities in the kidney sections as well as increased renal oxidative stress (MDA) and depletion of GSH levels. These derangements in renal physiology were also accompanied by activation of inflammatory signaling in renal tissues. Importantly, pretreating the experimental animals with piperine showed promising protective effects against IR-induced renal dysfunction, as evidenced by preserved renal function parameters, improved histopathology and improved tissue oxidative stress status as well as decreased activation of the inflammatory/apoptotic pathways.

The deleterious effects of IR are believed to be induced by tissue oxygen deprivation, which results in abnormal glucose metabolism leading to stopping of oxidative phosphorylation and ultimately depletion of cellular ATP.[3,26,29] Consequently, cellular anaerobic metabolism is encouraged, which does not provide enough ATP to support vital cellular enzymes such as the Na^+/K^+-ATPase with inevitable failure of mitochondrial as well as cellular membrane functions, not to mention the accumulation of lactate and intracellular acidosis.[30-32] Moreover, induction of oxidative stress, whether due to augmented production of reactive oxygen moieties or depletion of tissue endogenous antioxidant defense mechanisms, is a well-established mechanism leading to cellular

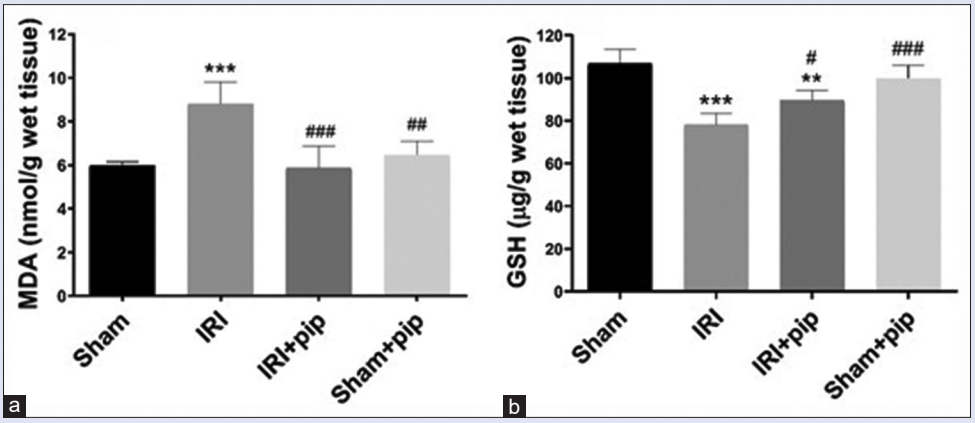

Figure 2: Effect of piperine on renal malondialdehyde (a) and reduced glutathione (b) in renal ischemia–reperfusion injury in rats. Values represent mean ± standard deviation ($n = 7$). $^{**}P < 0.01$, $^{***}P < 0.001$ versus the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus ischemia–reperfusion injury group. IRI: ischemia-reperfusion injury; Pip: Piperine

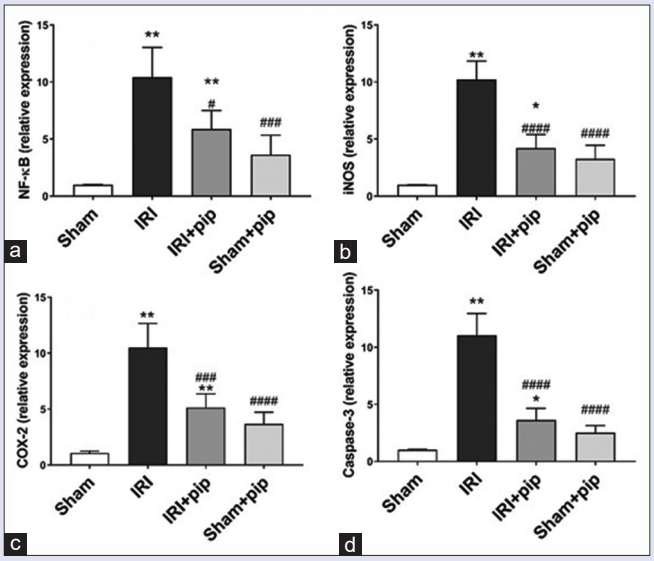

Figure 3: Effect of piperine on the expression of renal nuclear factor-kappa B (a), inducible nitric oxide synthase (b), cyclooxygenase-2 (c), and caspase-3 (d) in ischemia–reperfusion injured rats. Values represent mean ± standard deviation ($n = 7$). *$P < 0.05$, **$P < 0.01$ versus the sham group; #$P < 0.05$, ###$P < 0.001$, ####$P < 0.0001$ versus ischemia–reperfusion injury group. IRI: ischemia-reperfusion injury; Pip: Piperine

damage after IR.[3,6,13] In addition, previous studies illustrated that the induction of renal IRI upregulates inflammatory gene expression through activation of inflammatory cytokine production, notably tumor necrosis factor-α (TNF-α),[33] which delineates renal damage through induction of downstream signaling pathways including activation of NF-κB and its target genes as well as increased neutrophil activation resulting in increased oxidative stress, which in turn can add further insult to the kidney tissue.[29]

Induction of renal IRI in the current study increased oxidative stress in kidney tissues. This was manifested as increased levels of tissue MDA as well as diminished GSH content. Our findings are in accord with previous reports illustrating increased oxidative stress and oxidative stress-related damage induced by IRI in experimental animals.[13,26] Possible sources of increased oxidative stress in injured kidneys are thought to be increased neutrophil infiltration and increased myeloperoxidase activity,[8] in addition to the possible induction of iNOS, COX-2, and NADPH oxidase.[3,34] On the other hand, animals treated with piperine before induction of renal IRI showed lower levels of MDA and normalization of GSH, which agrees with previous reports showing potential antioxidant effects of piperine in a model of experimental arthritis.[18] Moreover, previous studies linked piperine treatment to decreased oxidative stress as well as enhanced activity of important endogenous antioxidant defense mechanisms including superoxide dismutase, GSH peroxidase, and catalase, all of which might contribute to increased bioavailability of GSH in situations where oxidative stress was induced by feeding animals a high-fat diet[35] or in diabetes-induced oxidative damage.[36]

The role of inflammation and activation of inflammatory signaling pathways is a well-established pathological mechanism during IRI.[3,6,8] The current results illustrated a robust increase in the expression levels of inflammatory (NF-κB, iNOS, and COX-2) as well as proapoptotic (caspase-3) genes subsequent to induction of renal injury by IR. These effects were mitigated by piperine pretreatment. These findings are supported by recent evidence showing that inflammation plays a key role in IR-related tissue injury.[37,38] The results introduced by Hu *et al.*[37] showed that activation of anti-inflammatory pathways resulted in better kidney function parameters, reduced tissue inflammation, and mitigation of oxidative stress in a rat model of kidney transplantation. Moreover, previous studies illustrated that increased inflammatory cytokines such as TNF-α is a common feature of IRI,[9] which can further induce activation of tissue inflammation and lipid peroxidation,[3,6,38] and contributes to increased failure of organ transplantation.[1,34] The antioxidant properties of piperine, at least in part, might be responsible for its ability to attenuate these inflammatory signals.[18,35,36] Moreover, the ability of piperine to antagonize inflammation, tissue infiltration, and remodeling was previously reported.[25] Induction of cerebral IRI in rats by Vaibhav *et al.*[21] resulted in tissue damage that was mediated via increased inflammatory cytokines and activation of NF-κB, iNOS, and COX-2. Importantly, their results showed that piperine treatment successfully mitigated these inflammatory effects and decreased neuronal cell death.[21] Similar anti-inflammatory effects of piperine were previously reported in other models of inflammation.[18,25,39-40] Recently, piperine administration for 10 days was shown to dose-dependently attenuate IR-induced acute kidney injury via antagonizing the increase of TNF-α and tissue oxidative stress.[24] Although these authors used lower doses (10 and 20 mg/kg/day) in a different rat strain (Wistar rats), their findings, nonetheless, support the results of the current study.[24] Importantly, the results of the current work not only illustrate the protective effects of piperine at a higher dose level (100 mg/kg/day) for a shorter duration (only 4 days compared to 10) but also highlighted the importance of other anti-inflammatory effects of piperine such as decreasing NF-κB, iNOS, COX-2 signaling as well its negative modulation of caspase-3.

Although piperine treatment in our study decreased the expression of the apoptotic factor caspase-3, which might contribute to its renoprotective effects, others reported that it might enhance the cytotoxic effects of chemotherapeutic agents[41] or have independent anticancer effects mediated via activation of apoptotic pathways.[42,43] On the other hand, some reports showed the involvement of the antiapoptotic effects of piperine in combating human keratinocyte damage induced by ultraviolet radiation,[44] which is in line with our findings. The possible explanation for this discrepancy between our results and others could be the difference in experimental settings as most of these studies were carried out on cells in culture,[41,42] while the current study was carried in an *in vivo* settings. Another explanation might be due to differences in duration of drug administration; for example, Yoo *et al.*[43] administered piperine to mice for 4 weeks as opposed to only 4 days in the current study.

CONCLUSION

The renoprotective effects illustrated by the results of the current study showed that piperine might be a promising candidate in the fight against renal IRI. These effects are, at least in part, mediated via the antioxidant and anti-inflammatory effects of piperine.

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Conflicts of interest

There are no conflicts of interest.

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