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EXPERIMENTAL STUDY

Protective effect of piperlongumine on inflammation and oxidative stress against ischemia-reperfusion injury in animal kidney

Zhenwen XIAO[1], Annamalai VIJAYALAKSHMI[2]

Department of Biochemistry, Rabiammal Ahamed Maideen College for Women, Thiruvarur, Tamil Nadu, India. **vijirajabio@gmail.com**

ABSTRACT

AIM: Piperlongumine (PL), an alkaloid from the *Piper longum* plant, is acknowledged for various biological properties. The study aimed to explore the protective effect of PL on ischemia reperfusion injury (I/R) in rat kidney.

METHODS. 24 adult male Sprague-Dawley rats (200 to 250 mg) were randomly allocated to four groups (n = 6/group): Group I: sham control, Group II: (I/R) renal ischemia/reperfusion kidney renal blocked for 1hour using clamps, followed by 2hr reperfusion. Group III: PL (25 µg/kg) + I/R group and Group IV: PL (50 µg/kg) + I/R group. Rat kidneys were exposed to 60 min of two-sided deep ischemia followed by 120 min of reperfusion. PL (25 and 50 µg/kg bw) was administered intraperitoneally half an hour before the ischemia. Creatinine, urea, and few renal markers activity in serum were assessed. Oxidative stress and inflammatory markers were also evaluated. In addition, the expressions of COX-2 and eNOS in animal kidneys were tested by western blotting.

RESULTS. Pre-treatment with PL in ischemia-reperfused rats significantly reduced the pathological damage in the kidney and declined the levels of serum creatinine and other renal parameters. PL treatment diminished the serum levels of TNF-α, IL-6, and IL-1β, as well as messenger RNA expressions. Important biological defence parameters such as superoxide dismutase and glutathione levels were upregulated while malondialdehyde levels were down-regulated in PL ischemia rats.

CONCLUSION. PL exhibits a protective effect against inflammation and oxidation in ischemia reperfusion animals *(Fig. 5, Ref. 32)*. Text in PDF *www.elis.sk*

KEY WORDS: piperlongumine, ischemia reperfusion, inflammation, oxidative stress.

Introduction

Ischemia signifies a lack of blood in tissues due to the hindrance of arterial flow. Reperfusion can unexpectedly worsen tissue damage and necrosis. Kidney injury is a critical disease condition. In developed countries, ischemia occurs mainly due to a clot in blood vessels or atherosclerosis. Significant risk factors are excessive tobacco smoking, lack of physical exercise, diabetes mellitus, obesity and hypertension. In some cases, a hereditary factor, male gender and aging are a ground for ischemia (1). Presently, there is no precise treatment for it. Renal ischemia-reperfusion damage is a complex effect resulting from vascular surgery and transplantation. Renal ischemia-reperfusion injury has been known as the main reason for severe renal failure (2). Clamping or compressing renal vessels and chilled environment condition also decrease the nutrients and oxygen supply to the renal tissue (3). Reperfusion of the ischemic kidney further deteriorates the oxidation and inflammation status, necrosis, cellular damage and cellular integration due to the production of pro-inflammatory mediators and release of reactive oxygen species (ROS) (4, 5). Pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) augment the generation of ROS. The inflammation plays a key role in the pathogenesis and progression of renal ischemia-reperfusion. Neutrophil penetration, increased TNF-α, IL-6, IL-1β, chemo attractant protein, and regular T cells enhance renal ischemia-reperfusion (6, 7). Elevated ROS leads to necrosis and programmed cell death by oxidative stress in cellular microenvironment. Consequently, downgrading the inflammation process and production of inflammatory intermediaries may effectively decrease the kidney injury (8). Additionally, renal ischemia reperfusion increases the formation of cyclooxygenase-2 synthase (COX-2), a key enzyme for inflammation, which augments the production of nitric oxide (NO) which reacts with ROS to constraint the endothelial nitric oxide synthase (eNOS) and vasoconstriction (9, 10). The biological effective compounds extracted from traditional medicinal plants

[1]Department of Nephrology, The Third Hospital of Jinan, Jinan, China, and [2]Department of Biochemistry, Rabiammal Ahamed Maideen College for Women, Thiruvarur, Tamil Nadu, India

Address for correspondence: Annamalai Vijayalakshmi, Department of Biochemistry, Rabiammal Ahamed Maideen College for Women, Thiruvarur, Tamil Nadu 610001, India.

containing antioxidants have been used as an alternative treatment due to their safety, convenience, and environment-friendly properties (11). Antioxidants have been shown to have protective efficacy against renal ischemia reperfusion injury and oxidative damage (12). The protective and healing effects of the Piperaceae family against various diseases including cancer have been researched and documented. Piperlongumine (PL), an alkaloid extracted from long pepper (*Piper longum* L.) exhibits anticancer effects in the lungs, breast and stomach by initiating a programmed cell death. Previous studies support our work in which PL management inhibits accumulated ROS inner cellular redox mechanisms (13). Anticancer properties of PL were explored in many studies (14, 15). In present study, the effects of PL on renal ischemia reperfusion were examined using an *in-vivo* experimental rat model. The role of ROS and the underlying intracellular mechanism were explored.

Materials and methods

Chemicals and reagents

Piperlongumine (Product No: SML0221) was purchased from Sigma-Aldrich (Mumbai, India). Biochemical markers, MDA (malondialdehyde) and GSH (glutathione) were purchased from FlukaChemical Corporation, NewYork, USA. To detect the activity of superoxide dismutase (SOD), a reliable assay kit (Calbiotech, ELIZA kits, Bioz, Incorporation, California, USA) was used. Results were determined by Shanghai Instrument Corporation, model UV-7200, UV-Visible spectrophotometer and microplate reader, Thermo Scientific, India. For kidney markers and serum markers, immunoassay kits were obtained from Neobioscience Cell Biolaboratory Incorporation, Shenzhen, China. COX-2, eNOS, and β-actin kits were obtained from ThermoFischer Invitrogen, India.

Animals and diet

Twenty-four adult male Sprague–Dawley rats (200 to 250 gm) were purchased from the Shandong Experimental Animal Center, Jinan, China. Animals were housed under regular light conditions (12 hr light/12 hr dark) at room temperature. Rats had free access to clean water and regular pellet feed. The animals were acclimated to lab conditions for one week. The present study followed the guidelines of the Medical University for utilizing lab animals for research purposes. Ethical approval was obtained from the Ethics Committee on Animal Experimentations of Shandong Hongli Medical Animal Experiment Research Co., Ltd (No. A21310058).

Experimental design

The rats were randomized in to 4 groups (n = 6/group) totally 24 animals. Group I: Sham control – The rats were given 0.5 ml saline intraperitoneally (i.p.) injections preceding the sham procedure in which the kidney was not clamped. Group II: (I/R) Renal Ischemia/Reperfusion kidney renal choked for 1 hr using clamps, followed by 2 hr reperfusion: The operation was performed in a similar form as group I. Group III: PL (25 μg/kg) + I/R group: Intraperitoneal PL was given 30 min preceding the initiation of ischemia. Group IV: PL (50 μg/kg) + I/R group: Intraperitoneal PL was given 30 min before surgical renal ischemia was initiated.

Experimental protocols and surgery

Prior to the surgery, animals were anaesthetized with isoflurane and positioned into a heating cloth to sustain body temperature at room temperature. Anaesthesia was continued with isoflurane (1.5 %) at 0.8 L/min. After midline laparotomy, pedicles of right and left kidneys were separated and clamped for 60 min in line with the standardized protocol (16). On termination of the reperfusion phase (120 min), the clamps were removed. The animal blood was collected through the carotid vein. Sham animals underwent the same operation without the clamping of renal pedicles.

Oxidative stress study of the kidney

To measure the oxidative stress in the kidneys, renal tissue homogenate was used to quantify MDA and GSH glutathione. Additionally, the homogenate mixture was further utilized to detect the activity of superoxide dismutase (SOD). To get accurate results, the manufacturer's guidelines for consistent assay kit were followed (Calbiotech, ELIZA kits, Bioz, Incorporation, California, USA). UV-visible spectrophotometer, Shanghai Instrument Corporation, model UV-7200 was used to read.

Determination of kidney markers

The crucial kidney markers were measured by regular standardized enzyme immunoassay kits (Neobioscience Cell Biolaboratory Incorporation, Shenzhen, China) to enumerate serum urea, creatinine and to further explore the serum absorptions of cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) (17).

RT-PCR analysis

PCR was used to assess levels of IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) in the kidneys. The entire cellular RNA was removed with TRIzol solution (SRL lab, China). Then, cDNA was produced with M-MLV (Moloney murine leukaemia virus enzyme) and buffer kit (Merck Darmstadt, Germany). The qPCR was executed by iProof master mixer (Bio-Rad, USA). The process was carried out in line with the manufacturer's protocol. The relative gene expression was analysed by comparative CT (−ΔΔCT) method.

Western blot analysis

Kidney tissues were homogenized in HEPES buffer (50mM) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), (50 mM) sodium fluoride, NaCl (40 mM), EDTA (1mM), triton X-100 (0.1 %), glycerol (5 %), sodium pyrophosphate (10 mM), β-glycerophosphate (10 mM) and 1.5 mM orthovanadate). Initially, homogenates were spun at 15,000 rpm for 15 min at 4 °C, and supernatants were separated. The Bradford method was used to quantify the total protein concentration (18). An amount of 50 μg of tissue proteins were filled and removed by PAGE and transported to immunoblot PVDF (polyvinylidene fluoride) membrane (Bio-Rad Lab Incorporation, USA). The membranes were blocked in skimmed milk (5 %) in Tris buffered saline-Tween buffer for 2 hours. Membranes were incubated at 37 °C for 1hour with equivalent secondary antibodies (1/20,000, HRP-conjugated anti-mouse). Then, the membranes were kept for characterization

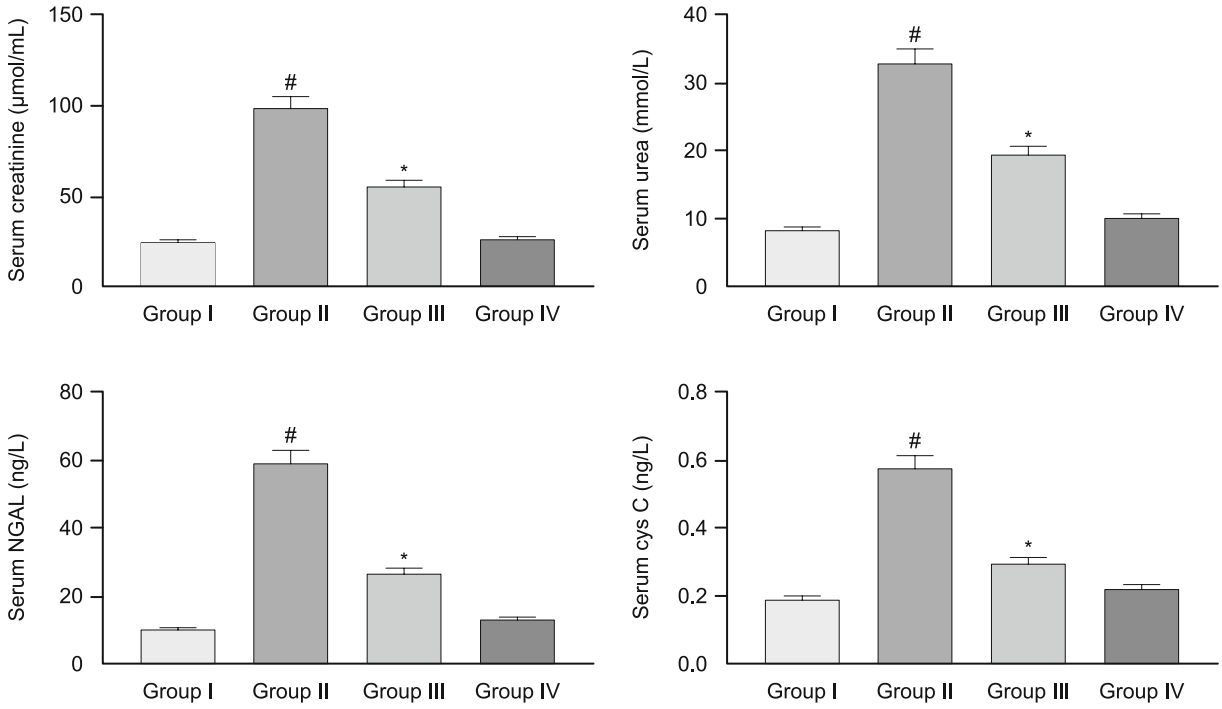

Fig. 1. Effect of piperlongumine on renal markers. Kidney function in animals subjected to the control sham protocol, and ischemia/reperfusion (I/R). Renal marker serum levels of creatinine, urea, neutrophil gelatinase-associated lipocalin, and cystatin C in the control, I/R and PL (25 or 50 µg/kg) + I/R groups. Results were expressed as mean ± SD (n = 6); * p < 0.05.

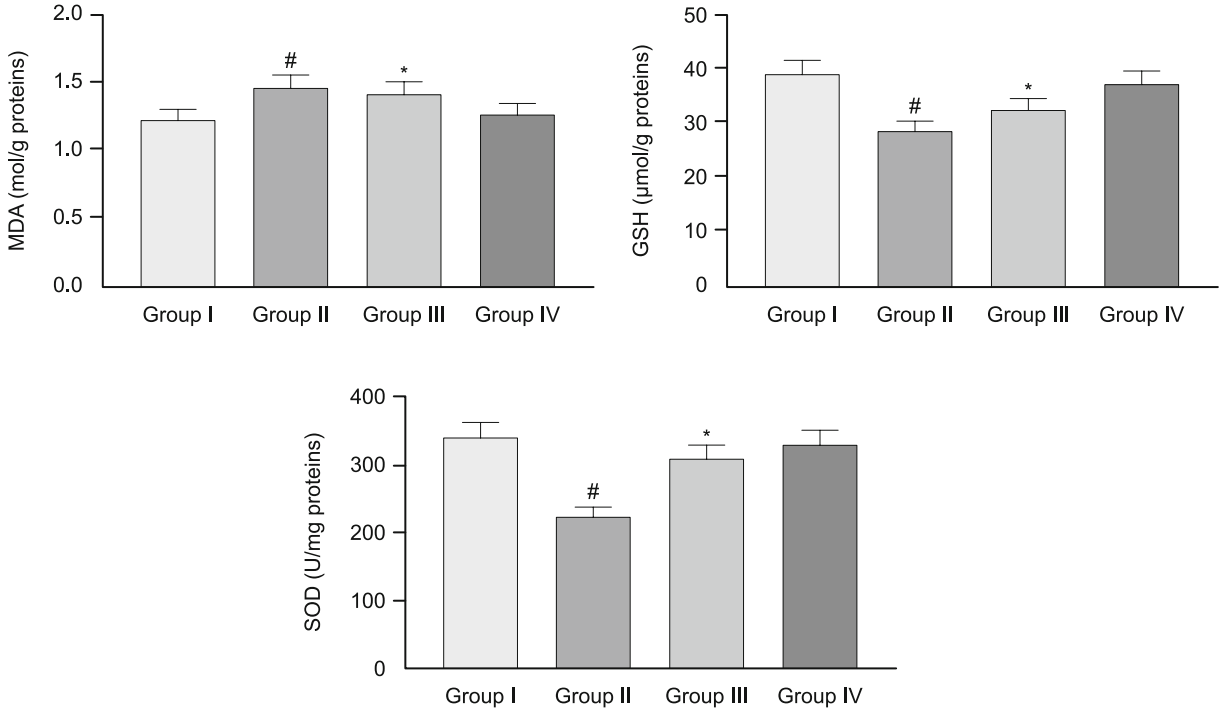

Fig. 2. Effect of piperlongumine on oxidative stress markers. Oxidative stress in animals exposed to sham protocol, and ischemia/reperfusion (I/R). Biochemical level of MDA, GSH and SOD in control, I/R, and PL (25 or 50 µg/kg) + I/R groups. Outcomes are expressed as mean ± SD (n = 6); * p < 0.05.

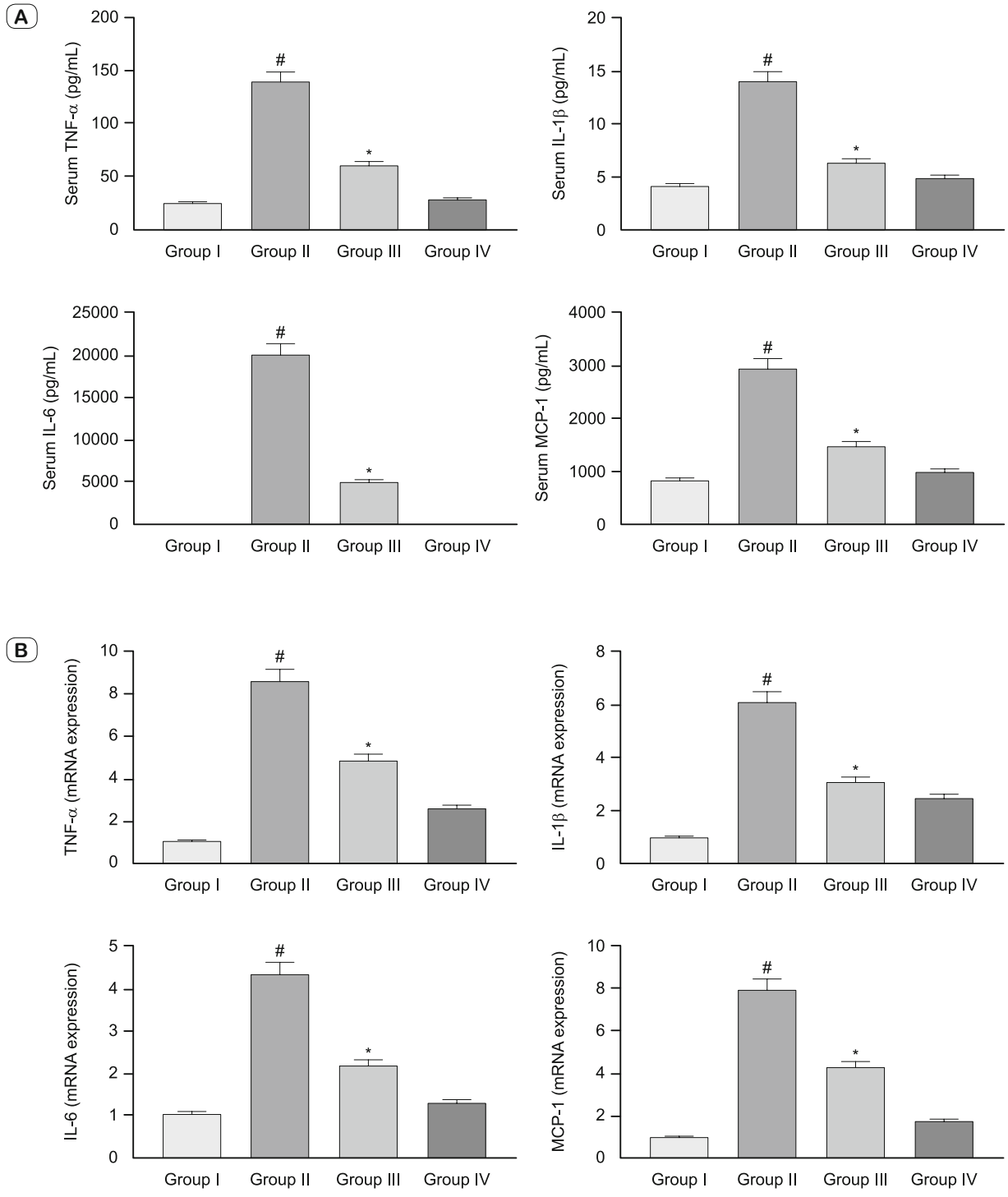

Fig. 3 (A–B). Effect of piperlongumine on inflammatory intermediate markers. The relative mRNA levels of the inflammatory mediators in kidneys of rats subjected to sham procedure and ischemia-reperfusion (I/R). RT-PCR trials of the messenger RNA levels of TNF-α, IL-1β, IL-6, and MCP-1 in renal tissues from normal rats, I/R & PL (25 or 50 μg/kg) + cluster. The levels of inflammatory mediators such as TNF-α in serum are described based on enzyme-linked immunosorbent assay. Further shows Interleukin (IL-1β, IL-6) and MCP-1 levels in the serum from the control , I/R and PL (25 or 50 μg/kg) + I/R groups. The results were expressed as mean ± SD (n = 6); * p < 0.05.

of protein for 12 hours at 4 °C with a specific antibody for every protein; COX-2 (Cayman Chemical Corporation, Michigan, United States), eNOS, and β-actin (Merck Ltd, Beijing, China). Finally, the protein band was noticed by enhanced chemiluminescence detection kit (Thermo Fisher Scientific, China).

Histopathological studies

The animals were sacrificed by cervical dislocation in accordance with the ethical protocol. The obtained kidneys were fixed in formalin (10 %), entrenched and segmented (2–3 μm), and stained with H&E (haematoxylin–eosin). Two pathologists independently studied the histological segments under a simple optical microscope (×400).

Statistical analysis

The data were expressed as mean ± standard error or proportion. Statistical analysis was done using GraphPad software v 6.0 using one-way analysis of variance (ANOVA) and Tukey's post hoc test. $p < 0.05$ was considered significant.

Results

Effect of piperlongumine on crucial kidney markers

A significant upregulation was noted in serum creatinine and urea suggestive of ischemia reperfusion (I/R) related to kidney disfunction in Group II rats. The upregulation was significantly ($p < 0.05$) downregulated in the PL-treated (25 and 50 μg/kg) rats in groups III and IV in comparison to the I/R rats in group II. This suggests that the renal function was significantly preserved with PL treatment ($p < 0.05$). Cystatin-C and neutrophil gelatinase-associated lipocalin levels in animals subjected to ischaemia reperfusion were significantly elevated as compared to sham control animals. Cystanin-C and neutrophil gelatinase-associated lipocalin levels were lowered in the PL-treated groups III and IV (25 and 50 μg/kg, respectively) animals as compared to I/R-induced rats in group II (Fig. 1).

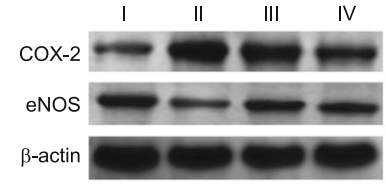

Fig. 4. Expression of COX-2, and eNOS protein markers in control and experimental rats. The expression of COX-2, and eNOS protein levels were measured by western blotting. β-actin was used as a loading control. Data are represented as mean ± standard error of the mean (n = 6).

Effect of piperlongumine on oxidative stress markers

The effect of PL on oxidative stress was also assessed. MDA in ischemia reperfusion animals was significantly elevated as compared to control rats (Fig. 2). PL treatment of rats in groups III and IV caused the oxidative stress levels to decrease as compared to ischemia reperfusion rats in group II. SOD and GSH levels in group II animals were significantly ($p < 0.05$) downregulated as compared to sham animals in group I. PL treatment (25 or 50 μg/kg) significantly ($p < 0.05$) upregulated SOD and GSH levels in animals from groups III and IV ($p < 0.05$).

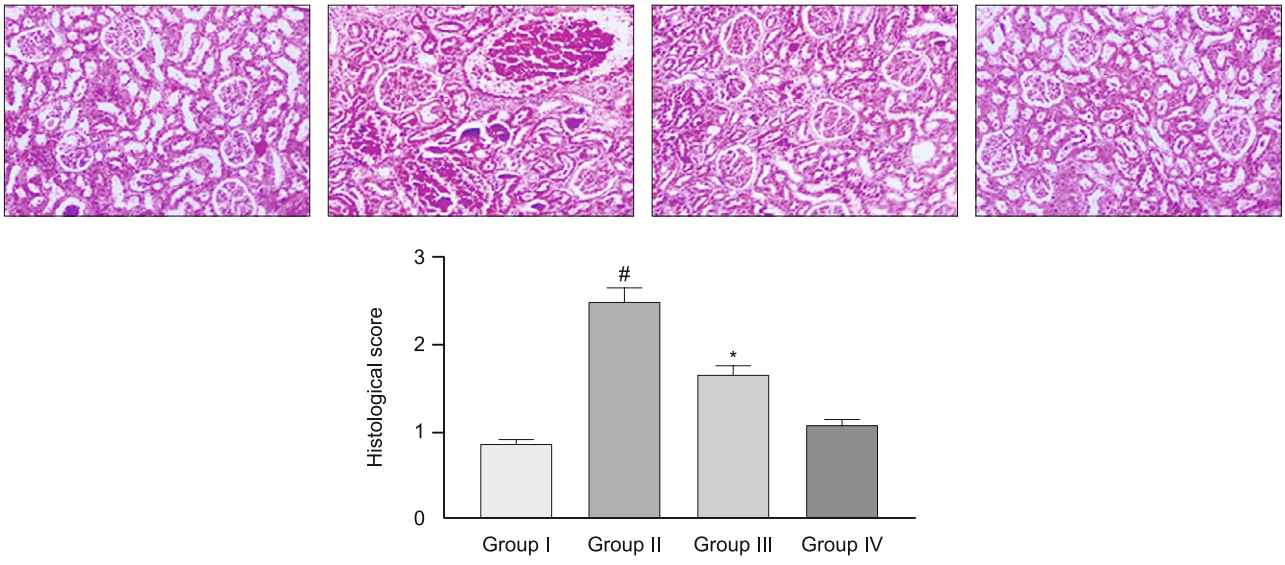

Fig. 5. Histological observation of renal tissues of rats. Histopathological examination (10x) showed piperlongumine treatment to have renoprotective efficacy. Kidney damage was imposed in the investigational cluster by compressing both renal arteries (ischemia/reperfusion (I/R). In the control group, both kidney pedicles were separated deprived of occlusion. PL was introduced 30 min prior to ischemia. Illustrative photographs were obtained from the sham control, I/R, PL (25 μg/kg) +I/R and PL (50 μg/kg) + I/R groups. Black arrows indicate the histological damage. Histological scores were quantified in I/R rats.

Effect of piperlongumine on intermediate inflammatory markers

The RT-PCR tested mRNA levels of TNF-α IL-1β, IL-6, and MCP-1 were significantly increased in the I/R group as compared to the sham control group (Fig. 3a–b). The levels of TNF-α, and inteleukins (1β, & 6) and MCP-1 (serum) in renal tissues of I/R animals were significantly increased ($p < 0.05$) as compared to PL-treated I/R rats. PL (25 or 50 μg/kg) has significantly downregulated ($p < 0.05$) the inflammatory marker levels as compared to I/R-persuaded rats. PL treatment had an inhibitory effect on inflammatory cytokines expression.

Effect of piperlongumine on COX-2 and eNOS

Expressions of crucial inflammatory markers, COX-2 and eNOS, were assessed. Sham control animals showed normal expression. I/R-induced animals in group II showed an upregulated expression of COX-2, and downregulated eNOS. The treatment with PL (25 or 50 μg/kg) downregulated the expression of COX-2 and downregulated eNOS significantly ($p < 0.05$) (Fig. 4). Thus, PL showed anti-inflammatory properties.

Effect of piperlongumine on histopathological observations

Figure 5 displays the histopathological pictures in which renal damage was observed. H&E-stained renal tissue was shown to be histologically damaged in I/R animals. Renal tissues in I/R animals developed focal tube-shaped necrosis, central renal haemorrhage, neutrophil penetration, and vacuolar deterioration of the renal tube-like epithelial cells. PL treatment reduced the pathological alterations in animals from groups III and IV as compared to I/R animals in group II. The histological changes were quantified and scored as described in Figure 5.

Discussion

Ischemia reperfusion injury is still a major complication of kidney transplantation and surgery. Numerous studies have recognized the multifaceted pathophysiological mechanisms in kidney ischemia/reperfusion. Researchers emphasized the role of numerous factors including oxidative pressure and inflammation in renal cells susceptible to injury. Therefore, various strategies have been developed to limit ischemic injury, such as usage of bioactive composites from plant source (19, 20). The present study established that piperlongumine protects the kidneys against ischemia/reperfusion-induced injuries in rat. Previous studies state that piperlongumine is a crucial phytochemical that exhibits numerous biological properties especially anticancer effect over melanoma, colorectal, and lung cancer cells. Many studies have reported the antioxidant, anti-inflammatory, antiarthritic, antimicrobial, antiasthmatic, antidiabetic, and immunomodulatory properties of piperlongumine (21). Piperlongumine showed hepatoprotective efficacy by downregulating the proportion of lipid peroxidation and elevated glutathione levels (22). Another experimental *in-vivo* study on rats verified the cardioprotective effect of piperlongumine as observed through histological and biochemical alterations (23). Anti-thrombogenic effects of piperlongumine (50 μg/kg) were also observed in the pulmonary coagulating mice model (24). However, to the best of our knowledge, the current study is the first research to explore the protective effect of piperlongumine on renal ischemia reperfusion injury. In the present work, serum creatinine, urea, neutrophil gelatinase-associated lipocalin and cystatin C were elevated with I/R. PL treatment downregulated the latter markers. Our results are supported by another study in which alkaloids showed a positive effect over serum markers such as neutrophil gelatinase-associated lipocalin, and cystatin-C (25). Thus, the kidneys face less oxidative pressure with PL administration.

The MDA level, a significant marker of oxidative injury, was downregulated. PL caused an increase in levels of antioxidants (SOD and GSH) as reported by another study (26). The findings propose that I/R reduced antioxidants due to elevated MDA and reduced GSH and SOD. This proposes that I/R might persuade oxidized tension and cause ischemic reperfusion injury. I/R injury also shows inflammatory changes (27). This alteration worsens the conditions due to the accumulation of immune cells, and inflammatory intermediaries to the tissues injured by ischemia/reperfusion (28, 29). Similar results were observed in the present study. Tumour necrosis factor-alpha, interleukins (1β, and 6) and MCP-1 were found elevated in the I/R group. In contrast, the PL treatment in groups III and IV lowered TNF-α, IL-1β, IL-6, and MCP-1 expressions as well as m-RNAs. Previous studies support our findings in which the disease development of I/R damage was predisposed by the inflammation-producing cytokines (30).

Subsequent to renal ischemia/reperfusion, the crucial inflammation markers such as COX-2 levels were increased in ischemia/reperfusion rats from group II. Nevertheless, the management with piperlongumine in groups III and IV decreased the COX-2 levels. The expression of eNOS was decreased in ischemia rats from group II. Treatment with piperlongumine in group III and IV upregulated the endothelial nitric oxide synthase. Similar findings of reduced eNOS in rats challenged with ischemia/reperfusion were seen in another study (31). In line withour study, other studies have proved through histological evidence that ischemic injury leading to kidney damage (neutrophil penetration, tube-shaped necrosis and renal haemorrhage) could be reversed by PL treatment (32).

Conclusion

Altogether the present study investigated to explined the protective effect of PL on ischemia-reperfusion injury (I/R) in rat kidneys through analysis the Oxidative stress markers, inflammatory markers and histopathological diagnosis and further study the mRNA expression of Inflammatory markers by RT-PCR methods, still this study were not investigated in previously. Our study validates the effectiveness of piperlongumine on ischemia-reperfusion rats with a positive effect. Piperlongumine administration in ischemic rats improves and protects against reperfusion by mitigating the inflammatory response and oxidative stress. The protective effect of piperlongumine was observed as a reversal of renal histopathologic damage in a rat model. Thus, piperlongumine is a potential preventive therapy for ischemic injury.

Future Directions

Numerous challenges exist in emerging diagnostic and molecular investigations of ischemia/reperfusion in humans. Animal models do not effectively imitate target attentions in human disease as well as pharmacokinetics, which is different too. Preclinical indication for the efficacy of PL in human models of renal I/R is essential. These studies should explore this matter in both genders with related comorbidities. Imaging analyses are essential for launching the clinical effectiveness of piperlongumine. Also, its preclinical safety must be assessed before it can be confirmed safe for humans, which can be nevertheless comfortably done with positron emission tomography examinations.

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