Zagazig University Medical Journal
www.zumj.journals.ekb.eg
https://doi.org/10.21608/zumj.2022.126747.2499 Volume 30, Issue 1, January 2024
Manuscript ID ZUMJ-2203-2499 (R2)
DOI 10.21608/ZUMJ.2022.126747.2499

Original Article

Diacerein protects against renal ischemia/reperfusion-induced kidney and lung injuries: involvement of osteopontin and Nrf-2/HO-1 pathway

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Submit Date 2022-03-14
Revise Date 2022-05-29
Accept Date 2022-06-20

ABSTRACT

Background: Renal ischemia/reperfusion is an imperative cause of acute kidney damage which in turn releases detrimental mediators into the circulation leading to subsequent remote organ injury.
Aim of the study: to assess the possible protective effects of diacerein (DIA), an interleukin-1 receptor antagonist, against renal ischemia/reperfusion (I/R) induced kidney and lung injuries in male albino rats.
Material, Methods: Rats were randomly assigned into: sham, I/R, and DIA-pretreated I/R groups. DIA was orally administered in doses of 25, 50 and 100 mg/kg/day for 20 days, then, both right and left renal pedicles were clamped for 45 min followed by reperfusion for 24 h. One-way ANOVA followed by Post-Hoc test were used to compare the results. P-Value of <0.05 was considered as statistically significant.
Results : DIA significantly reduced serum creatinine and blood urea nitrogen (BUN), improved both kidney and lung histoarchitecture, reduced pulmonary interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), osteopontin (OPN), elevated superoxide dismutase(SOD), glutathione (GSH), nuclear factor erythroid 2–related factor 2 (Nrf-2), Heme oxygenase 1 (HO-1), and reduced malondialdehyde (MDA) lung levels.
Conclusion: Our results show that DIA has a promising protective impact against kidney and lung injuries prompted by renal I/R. This effect is proposed to be mediated, partially, by downregulation of osteopontin and upregulation of Nrf-2/HO-1.

Keywords: diacerein, osteopontin, renal injury, lung injury

INTRODUCTION

Renal ischemia which is brought about by shock, surgery or transplantation, is a noteworthy cause of acute kidney injury (AKI) **[1].** Renal ischemic injury irreversibly damages peritubular vasculature causing hypoxia, that might be associated with the progression of chronic renal disease following AKI **[2]**.

The kidney is susceptible to ischemic injury due to its high basal o_2 demand, particularly the proximal tubule cells **[3]**. Although reperfusion is fundamental for ischemic tissue survival, reperfusion can cause additional cellular injury**.** Moreover, organs distant from the site of ischemia are subjected to injury following reperfusion of ischemic tissues e.g. liver, brain and the lung **[4].**

Ischemic tissues release chemokines that recruit inflammatory cells e.g. [neutrophils, dendritic cells, natural killer (NK)] to infiltrate the kidney tissues shortly after injury **[5],** in addition to expression of various cytokines e.g. [tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β)] **[6**].

Osteopontin (OPN), an acidic phosphoprotein, is expressed at low levels in normal kidneys and is upregulated in renal I/R **[7]**, hypertension, glomerulonephritis, ischemic acute renal failure and cisplatin-induced renal injury **[8].** OPN acts as a chemotactic factor by binding to αvβ3 integrin, CD44, collagen type I and fibronectin **[9]**. Moreover, OPN serves as a proinflammatory cytokine, enhances interferon gamma (IFN-γ) and IL-12 production but counteracts the antiinflammatory IL-10 production **[10].**

A previous study of **Zhang et al. [11]** proposed that OPN induces migration and activation of NK cells, augments ROS generation and boost NK-cell-mediated apoptosis of tubular epithelial cells in the kidney subjected to I/R.

Reactive oxygen species (ROS) are believed to have a crucial role in renal I/R injury **[12**]. ROS induce mitochondrial and plasma membrane lipid peroxidation leading to alteration of membrane structure and functions and cell injury **[13]**.

Diacerein is an anthraquinone that possesses anti-inflammatory, analgesic, and antipyretic properties which can effectively mitigate the symptoms of osteoarthritis **[14]**.

Aim of the work

The objective of this work was to address the protective effects of DIA on kidney and lung injury induced by renal I/R.

METHODS

Animals:The study was implemented on adult male albino rats weighing 200–250g, 8-10 weeks old obtained from the Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were housed at a room temperature (22 ± 2ºC), with a (12/12h) light/dark cycle and humidity (60±10%). The rats had free access to commercial pelleted rat chow and tap-water ad libitum. The study was approved by the institutional research board guidelines of the faculty of Medicine, Zagazig University, which follow the guidelines of the US National Institutes of Health of animal care (NIH Publications No. 8023, revised 1978).

Experimental design and dosing protocol: After acclimatization, rats were randomly divided into 5 groups (n = 6 each), where the Group I: sham group: received 2 ml saline solution by gastric gavage every day), Group II: I/R group, group III (DIA25), group IV(DIA50) and group V(DIA100): rats were pretreated with daily doses of diacerein dissolved in normal saline solution 0.9% and administered by gastric gavage in doses of 25,50,100 mg/kg respectively for 20 days **[15, 16]** and the I/R was induced on day 21.

Induction of renal I/R injury: Renal I/R was performed in rats as previously described **[17**]. Briefly, rats were anaesthetized with xylazine (10 mg/kg) and ketamine (75 mg/kg). After a midline abdominal incision both renal pedicles were isolated and bilateral renal ischemia was induced by placing a microvascular clamp over each pedicle for 45 min. At the end of the ischemic period, the clamps were removed and the abdominal incision was sutured and the rats were left to recover from anesthesia. The animals were kept well hydrated with saline and their body temperature was kept at 37ºC by placing the animals over a heating pad. The sham-operated group was subjected to the same surgical conditions without inducing I/R.

Laboratory analysis: At the end of the 24 hours reperfusion, animals were reanesthetized and blood was collected from the vena cava. Then, the kidneys and lungs were quickly removed **(18).** The left kidney and lung from each animal were kept in4% paraformaldehyde for histopathological assessment while the right lung was stored at -80˚C for lung tissue parameters estimations.

Renal function study: Serum was collected for biochemical assay of urea and creatinine. They were determined utilizing colorimetric diagnostic kits as per the manufacturer's directions **[15].**

Kidney histopathological study: Renal tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Then sectioned at 4 μm and stained by hematoxylin/eosin (H/E). Histological evaluation with light microscopy for kidney damage was conducted by a qualified pathologist who didn't have a clue about the groups. 10 fields of both outer medulla and cortex were randomly selected, and damage was scored according

to the percentage of injured tubules in the form of tubular necrosis and dilation, cast formation, loss of brush border and increased cell debris as summed up by Jablonski et al. [19]. Scores range from 0 to 4: 0 = no damage; 1 = less than 25% damage; 2= 25% -50% damage; 3 = 50%- 75% damage; and 4 = more than 75% damage.

Lung histopathological study: After sectioning of lung tissues into 5-µm thick, the sections were stained with H/E to be evaluated semiquantitatively under a light microscope. The severity of lung injury was determined according to Koksel's et al. [20] system on a scale from 0 to 3: grade 0, normal pulmonary architecture; grade1, mild to moderate interstitial congestion and neutrophil infiltrations; grade 2, perivascular edema, partial destruction of lung architecture and moderate neutrophil infiltration; and grade 3, severe destruction of the lung histology and massive neutrophil infiltration.

Assays of inflammatory markers (cytokine assay): Lung tissue TNF-α and IL-1β concentrations were measured in duplicate with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BioSource Europe S.A., Belgium) based on the manufacturer's instructions **[21]**. Results were expressed as pg/g tissue**.**

Estimation of plasma osteopontin: Blood samples were put in chilled tubes containing EDTA (2 mg/mL) and then centrifuged at 2400 g for 10 min at 4ºC. The plasma was frozen immediately and stored at -80ºC for ELISA. Plasma concentrations of OPN were measured utilizing a rat OPN ELISA kit (R&D Systems, Minneapolis, MN,USA) as per the manufacturer's instructions [22].

Immunohistochemistry:

After lung tissues had been processed and embedded in paraffin, 5 µm sections were cut and then stained immunohistochemically utilizing Ultrasensitive S-P and diaminobenzidine (DAB) staining kits (Maxin-Bio, Fuzhou, China). Immunohistochemical staining followed a basic indirect protocol utilizing a citrate antigen-retrieval method. The primary mouse monoclonal antibody against OPN protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1: 50. As a negative control, samples were incubated with 0.01 mol/L phosphate buffered saline (PBS) instead of the primary antibody. The OPN content was scored according to the spread of OPN immunoreactive cells: scale 0 = all cells are negative; 1= nearly 25% weakly positive cells; 2 = multifocal aggregates of uniformly stained cells, nearly 50%; 3 = diffuse positive staining throughout the cells, > 75% **[23].**

Evaluation of oxidant/anti-oxidant status: Levels of glutathione (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) activity in lung homogenates were assayed using commercial kits (Biodiagnostic,Giza, Egypt), based on manufacturer's instructions **[24].**

Real-time PCR analysis: Total RNA was extracted from lung tissue homogenate using RNA extraction kit (RNeasy FFPE Kit, Qiagen) following the manufacturer's instructions. Then, RNA was reverse transcribed using Quantiscript reverse transcriptase (Quanti Tect Reverse Transcription Kit, QIAGEN, Germany). PCR reaction was performed using SYBR Green PCR Master Mix (Applied Biosystems) and forward and reverse primers shown in table 1. Gene expressions for Nrf-2, HO-1 were expressed as fold change from the GAPDH level which is calculated as 2-ΔΔCt **(table 1).**

Compliance with ethical standards

The study was approved by the institutional research board guidelines of the faculty of Medicine, Zagazig University, which follow the guidelines of the US National Institutes of Health of animal care (NIH Publications No. 8023, revised 1978).

Statistical analysis:

All the results were expressed as mean ± SE. Statistical significance between various groups was tested utilizing one-way ANOVA followed by Post-Hoc (least significant difference "LSD") tests as described by Armitage and Berry [25]. Results were considered to be statistically significant when $P < 0.05$. Statistical package of social sciences (SPSS) computer software (version 16) was used to carry out the statistical analysis.

RESULTS

Effect of diacerein on kidney function biomarkers (serum creatinine and BUN):

Compared to sham group, renal I/R significantly ($p < 0.05$) increased serum levels of creatinine and BUN (725, 672%, respectively). Pretreatment with DIA (50 and 100 mg/kg) significantly

decreased the serum creatinine (-39,-63.6 %), while DIA (25, 50, 100 mg/kg) administration significantly reduced serum BUN (-17.8, -35.6 and -54% respectively) in relation to I/R group (figs.1A and 1B).

Effect of diacerein on kidney& lung histopathology:

The sham group displayed normal glomerular and tubular architectures while rats experienced I/R showed extensive acute tubular injury (necrosis, exfoliation of cells in the lumen, dilatation, loss of the brush border, cast formation and vascular congestion with a significantly high **Jablonski score)**. Gradual improvements in the renal tissue histopathology were detected in DIA treated groups which were associated with dose dependent decrease in the Jablonski score (Figs. 2A-F).The normal integrity of the pulmonary tissues was maintained in the sham group. Notably, disorganized alveolar structure was detected in I/R group, with a significant pulmonary interstitial edema, and inflammatory cells infiltrates with hemorrhage in the alveolar cavity. However, pretreatment with DIA mitigated lung injury in a dose dependent manner, as elucidated by intact alveolar integrity, regression in the interstitial edema, and a reduction in inflammatory cells infiltration (Figs. 3A-E) respectively). Histological scores corroborated these changes (Fig. 3F).

Effect on lung tissue inflammatory mediators:

Renal I/R significantly increased lung tissue IL- 1β, TNF- α (152, 156% respectively) as compared with sham group. A dose-dependent reduction was noticed with DIA pretreatment at 25, 50, 100 mg/kg in lung IL- 1β (-9, -36 and -53% respectively), TNFα (-15, -32 and -44% respectively) as compared with I/R group (Figs. 4A and B).

Effect on plasma osteopontin concentration:

Plasma OPN level in the various groups were measured to assess the role of OPN in renal I/R-induced lung injury. In the I/R group, plasma OPN concentrations increased significantly (260%) compared to sham group. DIA administration (25, 50 and 100 mg/kg) to rats underwent renal I/R significantly decreased plasma OPN level (-7, -22 and - 55% respectively) as compares with I/R group (Fig. 4C).

Immunohistochemical assessment of OPN immunoreactivity:

Concerning the expression of OPN protein, Immunohistochemically examined lung tissue showed significant extensive immunoreactivity of OPN in I/R group as compared to sham group that demonstrated no OPN immunoreaction. Administration of increasing doses of DIA (25, 50, and 100 mg/kg) resulted in gradual down regulation of OPN expression when compared with I/R group (Figs. 5A-F respectively).

Effect on pulmonary oxidative / antioxidative markers:

Renal I/R significantly increased lung expression of Nrf-2 and HO-1 (800 and 360% respectively), in addition to a significant elevation of MDA level (357%) compared to sham group. On the other hand, Renal I/R significantly decreased lung GSH level and SOD activity (-79, -65% respectively), compared to sham group. Administration of DIA (50 and100 mg/kg) significantly increased lung expression of Nrf-2 (106 and 189% respectively) and HO-1 (94 and 147% respectively) and decreased lung level of MDA (-41 and -59% respectively), in addition to a significant increase in lung GSH (100 and 196% respectively) and SOD (103 and 161% respectively) compared to I/R group (Figs. 6 A-E respectively).

Table.1. Forward and reverse primers used in RT-PCR experiment

gene	Primers
HO-1	Forward: 5′-GAG CCA GCC TGA ACT AGC-3′ Reverse: 5′-GAT GTG CAC CTC CTT GGT-3′.
Nrf-2	Forward: 5'-GAGACGGCCATGACTGAT-3' Reverse: 5'-GTGAGGGATCGATGAGTAA-3'
GAPDH	Forward: 5′-TGCTGGTGCTGAGTATGTCG- 3 Reverse: 5′-TTGAGAGCAATGCCAGCC- 3

HO-1, hemeoxygenease-1; Nrf-2, nuclear factor erythroid 2–related factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, housekeeping gene.

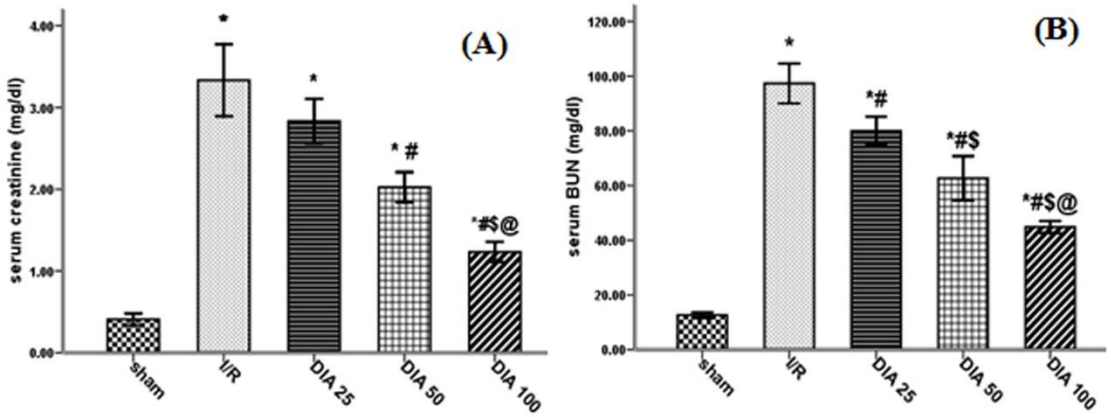

Fig. 1 : Effect of diacerein administration for 20 days (25 & 50& 100 mg/kg; DIA 25, DIA 50 & DIA100 respectively) on (A) the serum Creatinine, (B) BUN in rats subjected to ischemia (45 min)/reperfusion (24 h). Values are expressed as mean ± SE (n = 6). *P<0.05 compared to sham group, $^{\#}$ P<0.05 compared to I/R group, $^{\$}$ P<0.05 compared to DIA25, $^{@}$ P<0.05 compared to DIA50 , using one-way ANOVA followed by LSD post hoc test

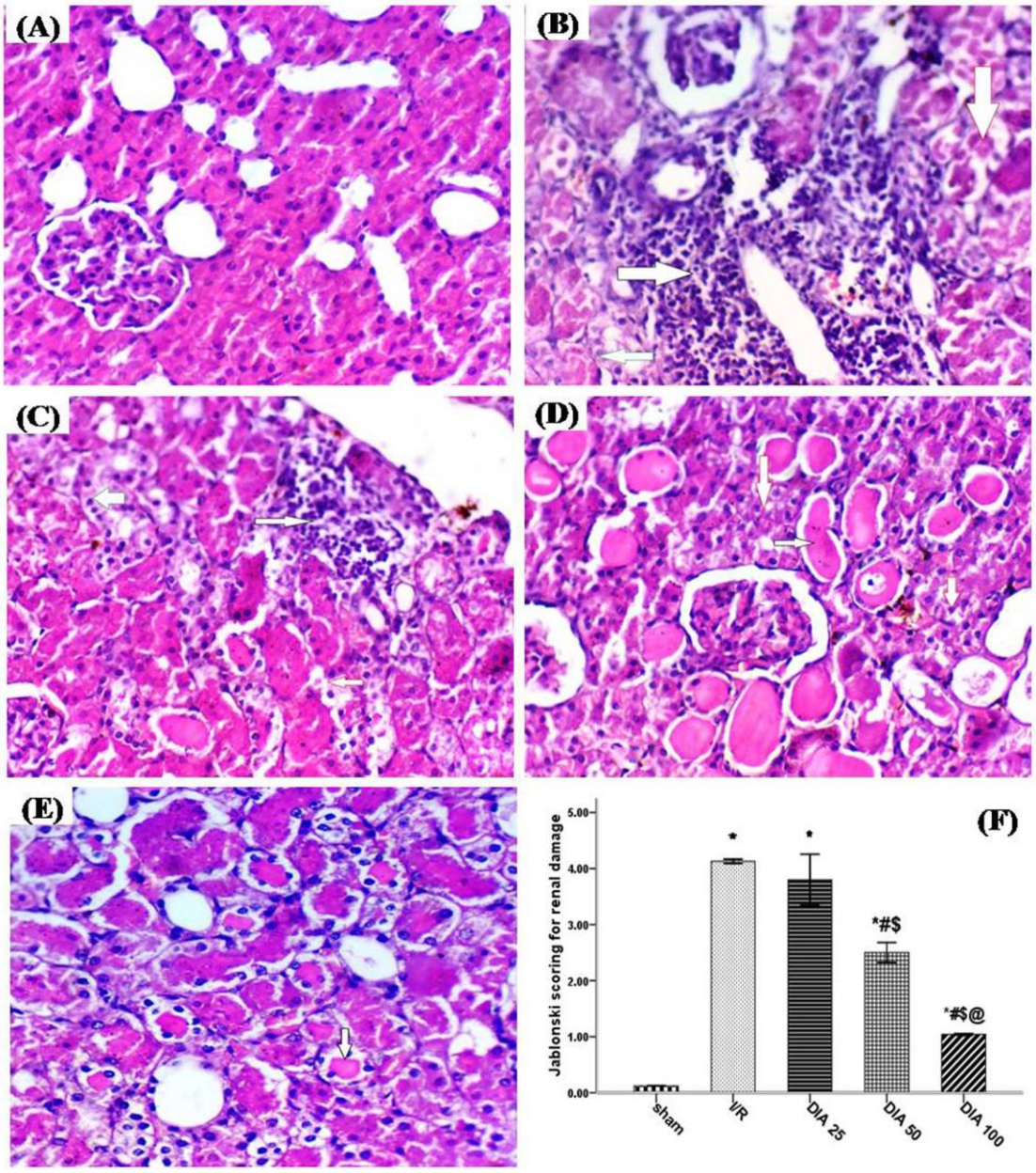

Fig. 2 Representative photomicrographs of renal tissues stained with H/E (x 400) and their Jablonski score in rats. **A** Sham rats showing normal kidney organization. **B** I/R group showing tubular cell necrosis and exfoliation of cells in the lumen (red arrow), cytoplasmic vacuoles (green arrow), marked inflammatory infiltrate (black arrow). **C DIA 25** section showing exfoliation of cells in the lumen (red arrow), cytoplasmic vacuoles (green arrow), moderate inflammatory infiltrate (black arrow). **D DIA 50** section showing mild cytoplasmic vacuoles (red arrow), tubular casts (black arrow). **E DIA 100** showing tubular casts (black arrow). **F** Average Jablonski scores for acute tubular damage from sham, I/R-untreated and I/R- DIA treated groups (DIA 25, DIA 50, DIA 100). Data were presented as mean ± SE (n = 6). $^{*}P<0.05$ compared to sham group, $^{\#}P<0.05$ compared to I/R group, $^{\$}P<0.05$ compared to DIA25, $^{@}P<0.05$ compared to DIA50, using one-way ANOVA followed by LSD post hoc test

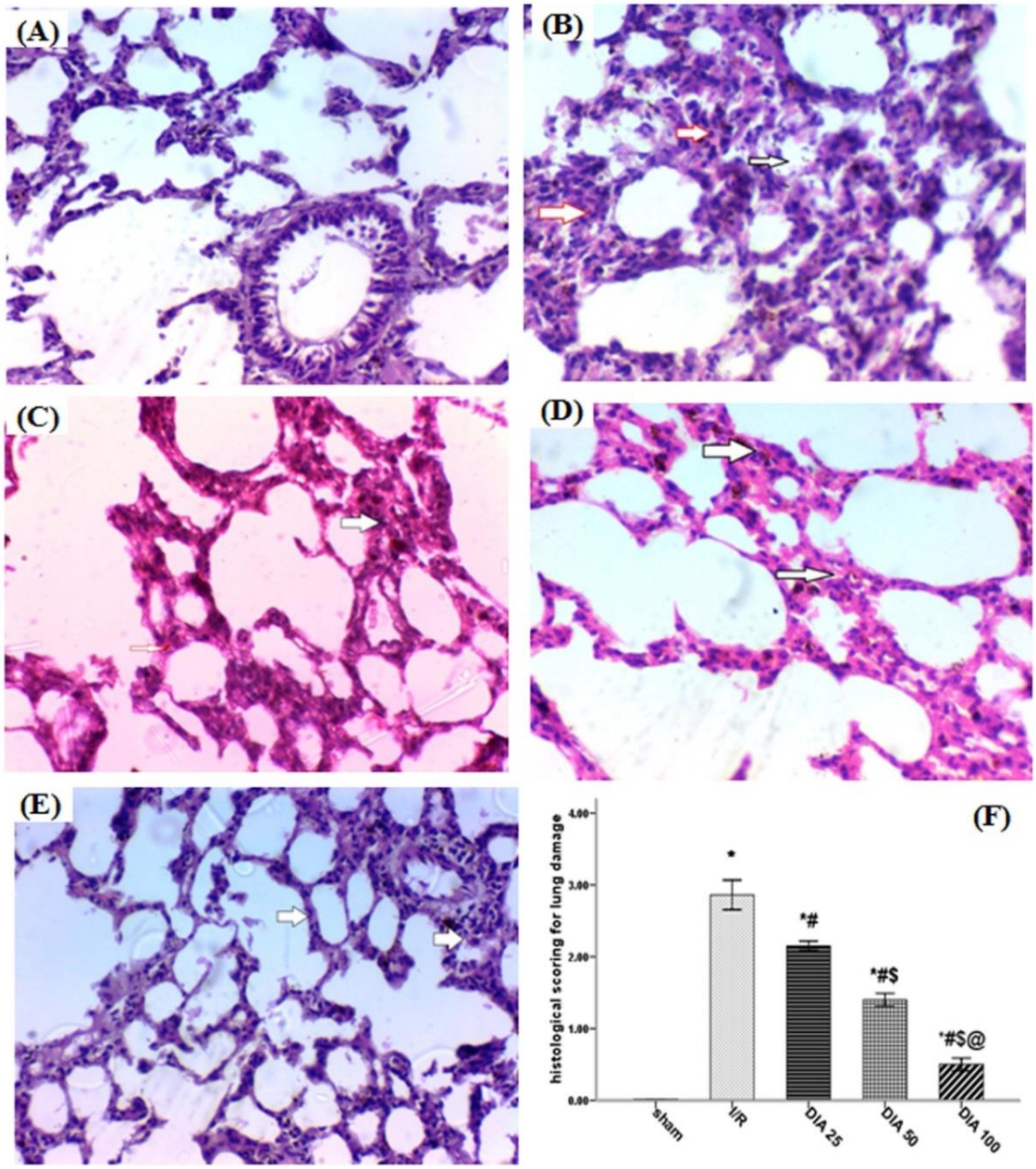

Fig. 3 representative photomicrographs of lung tissues stained with H/E (X400). **A** Sham rats showing normal pulmonary architecture. **B I/R** causes disorganized alveolar structure with a significant pulmonary interstitial edema (black arrow), inflammatory cells infiltrates with hemorrhage in the alveolar cavity, alveolar space disappearance and disintegration of lamina propria (red arrow). **C DIA 25**: showing moderate pulmonary interstitial edema and inflammatory cells infiltrates (black arrow) with hemorrhage (red arrow). **D DIA 50** showing mild pulmonary interstitial edema, and inflammatory cells infiltrates (black arrow). **E DIA 100** showing mild interstitial inflammatory cells infiltrates (black arrow). **F** Representative histogram for average pulmonary damage scores from sham, I/R-untreated and I/R- DIA treated groups (DIA 25, DIA 50, DIA 100). Values are expressed as mean ± SE (n = 6). *P<0.05 compared to sham group, $^{\#}$P<0.05 compared to I/R group, $^{\$}$P<0.05 compared to DIA25, $^{@}$P<0.05 compared to DIA50, using one-way ANOVA followed by LSD post hoc test

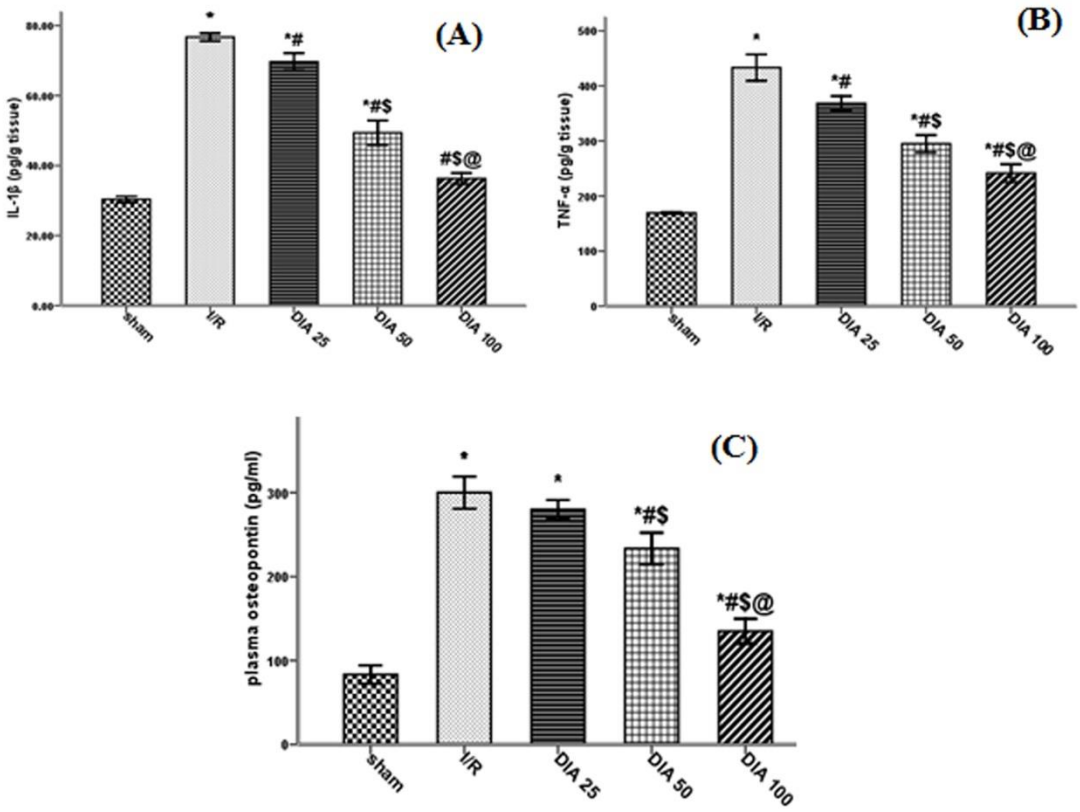

Fig. 4 Effect of diacerein administration for 20 days (25 & 50& 100 mg/kg; DIA 25, DIA 50 & DIA100) on **A** pulmonary IL-1β , **B** pulmonary TNF-α and **C** plasma OPN in rats subjected to ischemia (45 min)/reperfusion (24 h). Values are expressed as mean ± SE (n = 6). *P<0.05 compared to sham group, $^{\#}$P<0.05 compared to I/R group, $^{\$}$P<0.05 compared to DIA25, $^{@}$P<0.05 compared to DIA50, using one-way ANOVA followed by LSD post hoc test

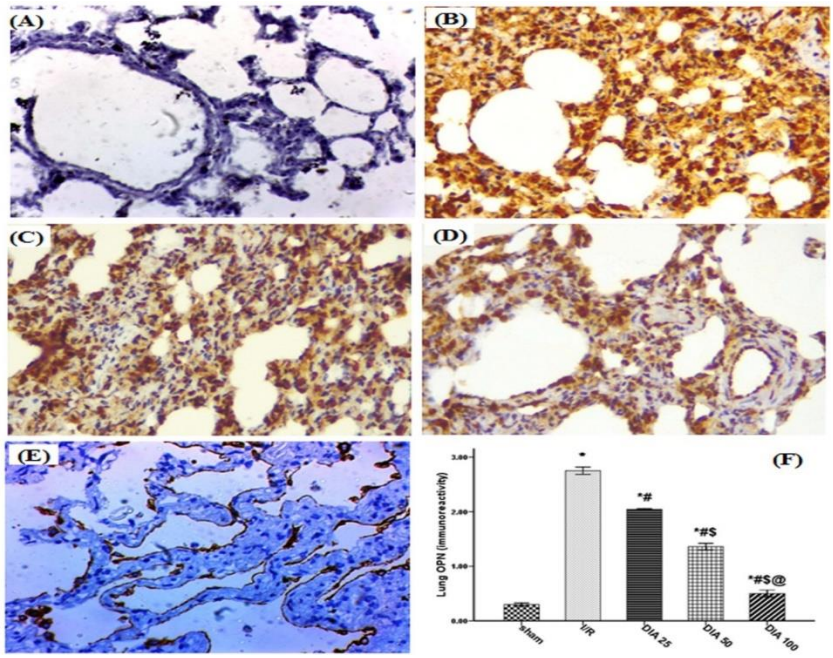

Fig. 5 Effect of diacerein administration for 20 days (25, 50& 100 mg/kg; DIA 25, DIA 50 & DIA100) on lung OPN expression using immunohistochemistry. Values are expressed as mean ± SE (n = 6). *P<0.05 compared to sham group, #P<0.05 compared to I/R group, $P<0.05 compared to DIA25, @P<0.05 compared to DIA50, using one-way ANOVA followed by LSD post hoc test

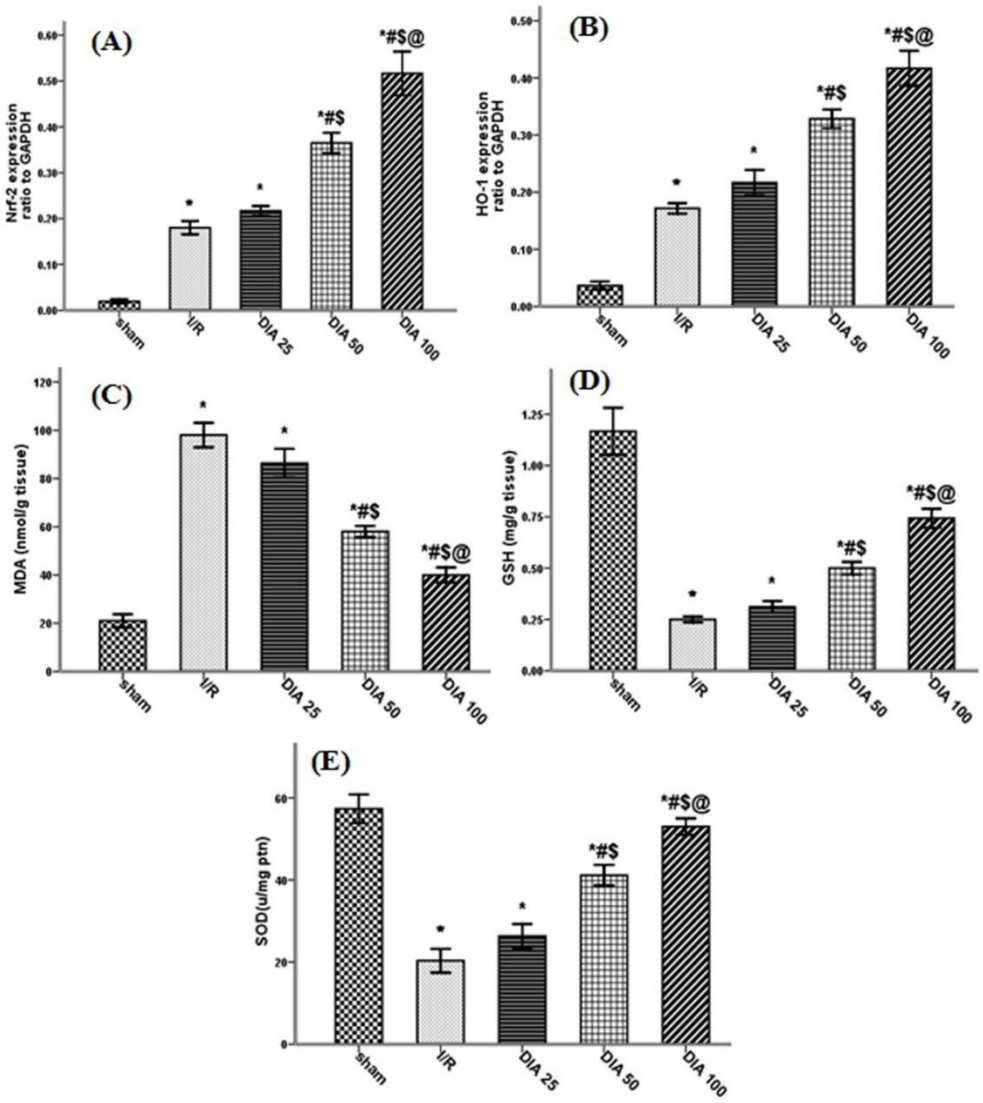

Fig. 6 Effect of DIA for 20 days (25, 50& 100 mg/kg/day; DIA 25, DIA 50 & DIA100) on the lung Nrf-2, HO-1 gene expression using RT- PCR (**A, B** respectively), **C** MDA content, **D** GSH content, **E** SOD enzyme activity in rats subjected to ischemia (45 min)/reperfusion (24 h). Values are expressed as mean ± SE (n = 6). *P<0.05 compared to sham group, #P<0.05 compared to I/R group, $P<0.05 compared to DIA25, @P<0.05 compared to DIA50, using one-way ANOVA followed by LSD post hoc test.

DISCUSSION

Renal I/R injury is a crucial clinical problem with high morbidity and mortality rates. Renal I/R injury involves direct cytotoxic effects of hypoxia, release of inflammatory cytokines, chemokines and other mediators **[26],** ROS generation**,** tubular cell apoptosis and mitochondrial dysfunction **[12].** The high death rates associated with AKI are commonly attributed to multi-organ failure including brain, heart, liver, intestine and lungs [**4**].

I/R-induced kidney injury is confirmed in this study by significant increase of serum creatinine and BUN levels in addition to toxic histopathological changes in the kidneys of the I/R group as compared to sham group. I/R is induced by ROS production that brought about neutrophil chemotaxis. Activated

neutrophils in turn release ROS, cytokines, proteases that enhance I/R injury. During reperfusion injury, further inflammatory mediators aggravate leukocytes activation and migration and subsequent multi-organ failure **[27]**.

In this study, lung injury following renal I/R was detected histopathologically by disorganized alveolar structure, significant pulmonary interstitial edema, inflammatory cells infiltrates with hemorrhage in the alveolar cavity when compared with sham group. The implication of inflammatory cytokines and ROS in lung injury following renal I/R was revealed in our study where IL-1β, TNF-α, and MDA were elevated while GSH and SOD levels were decreased in rat lungs. Those findings are in accordance with those of **Campanholle et al. [28].**

In our study, DIA pretreatment ameliorated toxic effect of renal I/R on rat kidneys and lungs evidenced by reduction of urea and creatinine level, in addition to improvement of kidney and lung histoarchitecture. The renoprotective effect DIA was previously reported by **Refaie et al. [15]** who studied the influence of DIA on doxorubicin induced kidney damage.

The pulmonary protective effect of DIA was further confirmed by reduction of lung inflammatory mediators IL-1β and TNF-α in a dose-dependent manner. These results cope with those of **Torina et al. [29].**

TNF-α is an inflammatory mediator that is supposed to have a fundamental role in I/R-induced injury not only to the ischemic organ but additionally to distant organs **[30]**. Various cells can produce TNF-α [e.g. macrophages, neutrophils, endothelial cells]. TNF-α acts locally in a paracrine fashion and, at distant sites, in a hormone like manner **[31].** TNF-α is associated with ROS production in glomerular cells and proximal tubular cells prompting renal injury **[16].** TNF-α is thought to stimulate other inflammatory mediator synthesis and neutrophils chemotaxis leading eventually to cell necrosis or apoptosis **[32].**

In line with our results, **Yousefi et al. [33]** observed that renal I/R increased TNF-α expression in the lungs of I/R group in comparison with the control group, which caused lung injury and necrosis.

Our study demonstrated that DIA pretreatment significantly decreased plasma as well as lung tissue OPN. OPN is a chemotactic factor, a potent inflammatory mediator, and a significant inducer of TNF-α, IFN-γ and IL-1β production at the inflammation sites **[10].**

In accordance with our study, **Cen et al. [7]**, used anti-OPN antibody to downregulate inflammatory cytokines expression in both kidney and serum. Interestingly, IL-1β was observed to be an intense and early stimulus for OPN expression by lung fibroblasts resulting in the secretion of soluble OPN protein **[34].** These facts explain our findings that DIA, as an IL-1β inhibitor, is supposed to ameliorate the lung insult provoked by renal I/R partially by opposing OPN expression and hence reduced lung inflammatory cells infiltration and inflammation.

Besides its anti-inflammatory effect, DIA opposed the oxidative stress- induced lung injury as it dose-dependently increased GSH and SOD while decreased MDA in the lung tissues in relation to I/R group. These effects coincide with those of **[16].**

Oxidative stress is a known pathogenic factor in AKI. During ischemia, $\mathbf{o_2}$ deprivation suppresses mitochondrial oxidative phosphorylation. Subsequently, ATP synthesis is impaired**,** and the activity of Na^+/ Ca^{+2} exchanger and Na^+/K^+/ATPase is suppressed leading to intracellular Ca^{+2} accumulation and activation of Ca^{+2}-dependent proteases that initiates cell apoptosis **[35]**. During the reperfusion phase, mitochondria will be subjected to excess Ca^{+2} and ROS which lead to progressive functional deterioration **[36]**.

Additionally, ROS stimulate inflammatory signaling leading to neutrophil sequestration in the kidney with upregulation of NF-κB and more production of TNF-α and inflammatory cytokines which further increase ROS production in a vicious cycle **[16].** Overproduction of ROS overwhelms the endogenous antioxidants and induces peroxidation of cell membrane unsaturated fatty acids with subsequent cell lysis. ROS-induced peroxidation can damage tissue lipids, proteins, and DNA **[37].**

In accordance with our study, **Tu et al. [38],** postulated that rhein, the active metabolite of DIA expressed a protective effect on adenine- induced renal injury.

In our study, DIA was found to increase Nrf-2/HO-1 expression in lung tissues. HO-1, a pivotal cellular antioxidant enzyme**,** can attenuate oxidative stress, reduce inflammatory response, and decrease the rate of apoptosis**.** On the other hand, HO-1 expression is suppressed by IL-1β, IL-17 and TNF-α **[39].** The beneficial role of HO-1 was studied in different experimental disease models such as atherosclerosis, cardiac I/R injury, diabetes, inflammatory bowel disease or rheumatoid arthritis **[40]**.

Nrf-2 is a transcription factor that regulates HO-1 gene expression via binding to antioxidant response element (ARE)-dependent genes **[41].** Thus, the increase of Nrf-2/HO-1 expression in lung tissues observed in DIA pretreatment groups could also partially explain the DIA pulmonary protective effect.

Conclusion

In conclusion, the present study supposes DIA as a potential protective drug against renal I/R induced kidney and lung injuries and suggested the implication of osteopontin and Nrf-2/HO-1 in mediating this effect. In addition, this study should contribute to the development of new studies focusing on DIA as a therapeutic target for preventing renal or lung injury following renal ischemia resulting during surgery or transplantation.

***Conflicts of interest*:** no conflicts of interest
***Financial disclosures*:** no disclosures

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Acknowledgement:

The authors acknowledge Dr. Hayam E. Rashed, Pathology Department, Faculty of Medicine, Zagizag University for her assistance in histopathological examination.

To Cite :

Elwany, N., Ahmed, H., Mahmoud, N. Diacerein protects against renal ischemia/reperfusion-induced kidney and lung injuries: involvement of osteopontin and Nrf-2/HO-1 pathway.. *Zagazig University Medical Journal*, 2024; (14-23): -. doi: 10.21608/zumj.2022.126747.2499