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Pioglitazone modulates immune activation and ameliorates inflammation induced by injured renal tubular epithelial cells via PPARγ/miRNA-124/STAT3 signaling

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Abstract. Acute kidney injury (AKI) is commonly a result of renal ischemia reperfusion injury (IRI), which produces clinical complications characterized by the rapid deterioration of renal function, leading to chronic kidney disease and increases the risk of morbidity and mortality. Currently, only supportive treatment is available. AKI, which is accompanied by immune activation and inflammation, is caused by proximal tubular injury. The present study investigated the role of tubular epithelial cells as drivers of inflammation in renal IRI and their potential function as antigen-presenting cells, as well as the molecular mechanisms by which peroxisome proliferator-activated receptor-γ (PPARγ) agonists [such as pioglitazone (Pio)] exert reno-protective action in renal IRI. A total of 50 Wistar male albino rats were divided into five groups: Sham + DMSO, Sham + Pio, IRI + DMSO, IRI + prophylactic preoperative (pre) Pio and IRI + postoperative Pio. The histopathological changes in renal tissue samples and the renal epithelial cell expression of CD86, miRNA-124, STAT3, pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and Arginase-II were analyzed by immunohistochemistry, reverse transcription-quantitative PCR, western blotting and ELISA respectively. IRI was a potent inducer for CD86 immunoexpression. An ameliorative action of Pio was demonstrated via decreased CD86 immunoexpression, upregulation of miRNA-124, decreased STAT3 expression and beneficial anti-inflammatory effects. The tubular epithelium served a notable role in the inflammatory response in renal IRI. Pio exerted its anti-inflammatory effects via PPARγ/miRNA-124/STAT3 signaling.

Introduction

Renal ischemia reperfusion (IR) often results in acute kidney injury (AKI), a clinical condition with no effective treatment, which increases the risk of morbidity and mortality perioperatively (1,2). Because of excessive workload and greater metabolic demand, as well as limited anaerobic energy production, proximal S3 segment tubular epithelial cells (TECs) of the outer medulla are most commonly affected by acute ischemic injury (3). The unique microvasculature of this structure makes it vulnerable to renal hypoxia, hypoperfusion and mitochondrial damage (4).

Inflammation is associated with the pathophysiology of renal IR injury (IRI) (5). Following ischemic injury, endothelial cells and leukocytes serve a role in initiating inflammation and damaged TECs contribute to the inflammatory process. Injured tubular epithelium produces numerous cytokines including IL-6, IL-1β, TNF-α and TGF-β, thereby affecting the behavior of macrophages and inducing a pro-inflammatory phenotype (3,6,7). To the best of our knowledge, it has not been determined whether renal epithelial cells serve as antigen-presenting cells (APCs) and exert an immunomodulatory function during renal IR.

During inflammation, inducible nitric oxide synthase (iNOS) is upregulated and converts arginine into citrulline and NO (8). This enzyme is found in the renal tubules, interlobar and arcuate arteries and glomerulus of normal rat kidney (9). Studies have documented the involvement of iNOS and NO in renal IRI development and suggested that iNOS inhibitors may prove beneficial as a therapeutic strategy in clinical scenarios where renal IRI is prevalent (10,11). Arginase-II (Arg-II), which is highly expressed within the S3 proximal TECs (12), catalyzes the conversion of L-arginine to L-ornithine and urea, which is needed for the synthesis of polyamines (13).

Since iNOS and Arg-II use the same substrate, stimulating Arg-II expression exerts anti-inflammatory effects via shifting of arginine metabolism to produce polyamine at the expense of NO production (14).

Signal transducer and activator of transcription 3 (STAT3) was identified by studies on acute response factor signaling (15,16). During the binding of cytokines, JAK protein stimulates canonical STAT3 signaling. The most common activators of STAT3 are IL-6-type cytokines via IL-6-induced tyrosine phosphorylation of STAT3 (17). Dysregulation in the activation of STAT3 is typically associated with multiple pathologies, including autoimmune and malignant disorders (18). The role of STAT3 in the progression of diabetic nephropathy, development of HIV-associated nephropathy, activation of renal interstitial fibroblasts and progression of renal fibrosis has been investigated (19-21). Numerous studies have also noted an association between IRI progression and the activation of STAT3 (22,23), some of which found that activation of STAT3 in renal proximal TECs may be protective during IRI (24,25). Although there is limited data regarding the therapeutic potential of STAT3 inhibitors in pathological renal models, evidence suggests that STAT3 inhibitors may be beneficial (26,27).

Peroxisome proliferator-activated receptor (PPAR)γ, a nuclear receptor superfamily member, is a transcription factor involved in regulating glucose and lipid metabolism as well as cancer progression and inflammation (28). PPARγ agonists [such as pioglitazone (Pio)] inhibit inflammation by stopping the phosphorylation of proteins involved in JAK-STAT signaling pathway (29,30). PPARγ binds to miR-124 promoter, causing the upregulation of miR-124 (31), thereby regulating gene expression. Sun *et al* (32) reported that miR-124 targets STAT3 to decrease the production IL-6 and TNF-α converting enzyme to decrease TNF-α release.

More studies are required to understand the inflammatory response mechanisms during ischemic kidney injury to identify the molecular targets for therapeutic intervention. The present study aimed to determine the role of renal TECs as drivers of inflammation in renal IRI and their potential function as antigen-presenting cells by analyzing inflammatory markers involved in pathogenesis of renal IRI, as well as the renal epithelial cell expression of CD86, STAT3 expression in renal IRI and the molecular basis underlying the anti-inflammatory action of the PPARγ agonist Pio by investigating its effect on the expression of miRNA-124, STAT3, pro-inflammatory cytokines, iNOS, Arg-II and CD86.

Materials and methods

Chemicals and reagents. Pio was purchased from Arab Pharmaceutical Manufacturing Co., Ltd. Dimethyl sulfoxide (DMSO) was purchased from Loba Chemie Pvt. Ltd.

Animals. A total of 50 adult Wistar male albino rats (age, 6-8 weeks; weight, 160-180 g) were obtained from the Faculty of Agriculture, Benha University, Moshtohor, Egypt. Animals were randomly divided into five groups (all n=10) and each group was placed in a separate cage. The cages were maintained at 25˚C with 12/12-h light and dark cycles, relative humidity (45±5%) and all animals had

access to food and water *ad libitum*. All rats were acclimatized to the laboratory setting for one week prior to experiments. The study followed the criteria of care and use of laboratory animals (33) and was approved by the Medical Research Ethics Committee of Benha University, Egypt (approval no. RC.11.6. 2022).

Rat model of renal IRI. The animals were divided into the following groups: i) Sham operation + DMSO; ii) sham operation + Pio; iii) renal IRI + DMSO; iv) IRI + prophylactic preoperative (pre) Pio and v) IRI + postoperative (post) Pio. All rats were anesthetized using Thiopental Na [40 mg/kg, administered intraperitoneally (i.p.)] and injected intramuscularly with antibiotic (Penicillin G procaine; 40,000 U/kg). Renal IR was performed by clamping the renal arteries bilaterally for 45 min, followed by reperfusion for 24 h, as described by Hu *et al* (34). Rats in sham operation groups underwent similar surgical interventions and were anesthetized but did not undergo bilateral renal pedicle clamping. Pio was dissolved in DMSO and injected i.p (10 mg/kg) as previously described (35). The drug was administered 2 h before sham operation or induction of ischemia in groups II and IV respectively, and 2 h after surgery in the IRI + postoperative (post) Pio group. Respiratory rate and pattern of rats was monitored every 10-15 min and rats were turned from side to side during the recovery period to promote a quicker recovery. Food and water intake was also monitored after recovery. At 24 h post-reperfusion, rats were euthanized via decapitation following anesthetization with 1.5 g/kg urethane (i.p). Death was verified by cessation of heartbeat and respiration, then bilateral nephrectomy was performed and each kidney was cut into two.

Renal function assessment. Blood samples (2 ml) taken from the abdominal aorta, 24 h after reperfusion, were left to clot for 15-30 min at room temperature, centrifuged at 3,000 x g at 4˚C for 10 min and supernatant was obtained to monitor renal function. Serum creatinine and blood urea nitrogen (BUN) levels were estimated using Rat Creatinine (cat. no. #MBS749827) and BUN ELISA kits (cat. no. #MBS2611086; both MyBioSource, Inc.), according to the manufacturer's instructions.

Biochemical analysis. The kidney specimens were rinsed in ice cold saline and homogenized using a Mixer Mill MM400 (Retsch GmbH) in phosphate buffer (pH 6-7). Tissue homogenate was centrifuged at 10,000 x g, 4˚C for 15 min. Supernatant was used for quantitative detection using ELISA kits, according to the manufacturer's instructions, as follows: Rat IL-1β (cat. no. E-EL-R0012; Elabscience Biotechnology, Inc.), IL-6 (cat. no. ab100772; Abcam), TNF-α (cat. no. E-CL-R0019) and TGF-β1 (cat. no. E-EL-0162; both Elabscience Biotechnology, Inc.), Arg-II (cat. no. MBS7216305) and iNOS ELISA kit (cat. no. MBS023874; both MyBioSource, Inc.).

Histopathological examination. The kidney samples were fixed in 10% buffered formalin (pH 7.8) for 72 h at room temperature, then sliced into very thin sections (4 *μ*m), stained with hematoxylin and eosin and visualized using the high-power option of the light microscope (magnification, x400). Histopathological samples were scored using the system

described by El-Nabarawy *et al* (36) as follows: -, no abnormal cellularity; +, minor focal lesions in 1-3 samples/group; ++, mild focal lesions in 4-6 samples/group; +++, moderate diffuse lesions in 4-6 samples/group and ++++, severe diffuse lesions in all samples.

Immunohistochemistry staining. Deparaffinized, rehydrated 4-μm tissue sections in descending alcohol series at room temperature were subjected to antigen-retrieval at 95˚C, then blocked by 0.3% H_2O_2 for 20 min at room temperature. Sections were incubated with anti-CD86 primary antibody (cat. no. bs-1035R; BIOSS USA; 1:150) overnight at 4˚C, washed with PBS, then incubated with secondary antibody HRP Envision kit (Dako; Agilent Technologies, Inc.) for 20 min and DAB for 15 min. Sections were washed with PBS, counterstained with hematoxylin, dehydrated and cleared in xylene and finally cover slipped for microscopic examination. A total of six non-overlapping fields were randomly selected and scanned from each sample for the determination of mean area percentage of immunohisto-chemical expression levels of CD86 positive cells. All light microscopic examination and morphometric data were obtained using Leica Application module for histological analysis attached to Full HD microscopic imaging system (Leica Microsystems GmbH).

Western blot analysis. Western blotting was performed to detect STAT3 expression levels. Total protein was extracted using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) and protein concentration was determined colorimetrically in kidney tissue samples using the Bradford method (37). A total of 25 μg protein/lane was mixed and boiled with SDS Loading buffer for 5 min. The solution was left to cool on ice for 7 min before loading into a 10% SDS-polyacrylamide gel and separated using the Cleaver electrophoresis unit (Cleaver Scientific Ltd., UK) and placed on PVDF membranes for 30 min via Semi-dry Electroblotter (Bio-Rad Laboratories, Inc.). Blocking was performed with 5% non-fat dry milk in Tris-buffered saline-0.05% Tween-20 (TBS-T), for 2 h at 37˚C. Incubation of the membrane was performed overnight at 4˚C with primary antibodies against STAT-3 (1:500; cat. no. ab119352; Abcam) and β-actin (1:500; cat. no. A5060; Sigma-Aldrich; Merck KGaA). Blots were washed three times (10 min each) using TBS-T, incubated at room temperature for 1 h using horseradish peroxidase-linked secondary antibodies (Dako; Agilent Technologies, Inc.), then washed three times (10 min each) with TBS-T. Chemiluminescent Western ECL substrate (PerkinElmer, Inc.) was applied according to the manufacturer's guidelines. Signals were captured using the Chemi Doc imager (Bio-Rad Laboratories, Inc.). Band intensity was normalized to β-actin.

Reverse transcription-quantitative (RT-q)PCR analysis of miRNA-124 gene expression. Total RNA was extracted from frozen kidney tissue samples using TRIzol™ Plus RNA Purification kit (cat. no. 12183555; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's guidelines. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific,

Inc.). Pure RNA has A260/A280 ratio of 1.8-2.1 (38). Rat rno-mir-124 Real-time RT-PCR Detection and U6 Calibration kit (cat. no. MBS826191; MyBioSource, Inc.) was used for the detection and quantification of mir-124. RT was performed according to the Standard RT Reaction Program (30 min at 25˚C, 30 min at 42˚C, 5 min at 85˚C) followed by PCR reaction (95˚C for 3 min hold, 40 cycles of 95˚C, 12 sec; 62˚C, 40 sec) using Step One Plus Real-Time PCR System (Thermo Fisher Scientific, Inc.). The relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method described by Livak and Schmittgen (39). The results are expressed as the fold-change relative to the Sham operation + DMSO group.

Statistical analysis. Data are presented as the mean ± SD. Differences between groups were evaluated using one-way ANOVA followed by post hoc Tukey's test using Statistical Package for Social Science program, Version 16 (SPSS, Inc.). P≤0.05 was considered to indicate a statistically significant difference.

Results

Effect of Pio on serum levels of creatinine and BUN. Serum creatinine and BUN levels at 24 h after reperfusion were significantly increased in the renal IRI + DMSO group compared with the sham groups (Sham operation + DMSO, Sham operation + Pio). Administration of Pio prior to isch-emia induction did not cause a significant decrease in serum creatinine and BUN levels compared with the renal IRI + DMSO, while its administration in the post-IR phase caused a significant decrease in the serum creatinine and BUN levels compared with the renal IRI + DMSO and the group adminis-tered Pio prior to ischemia induction (Fig. 1A and B).

Effect of Pio on iNOS, Arg-II and proinflammatory cytokines levels. In the renal IRI + DMSO group, levels of pro-inflammatory cytokines (IL-6, IL-1 β and TNF-α), TGF-β and iNOS were significantly increased compared with the Sham groups (Sham operation + DMSO, Sham operation + Pio) (P<0.05; Table I). Administration of Pio prior to isch-emia or post-IR caused a significant decrease in all assessed pro-inflammatory cytokines as well as iNOS levels compared with the IRI + DMSO group. A significant decrease in IL-1 β and iNOS levels was detected when Pio was administered in post-IR compared with administration before induction of ischemia. A significant decrease in Arg-II was demonstrated in the renal IRI + DMSO group compared with the sham groups (Sham operation + DMSO, Sham operation + Pio), while Pio administration prior to ischemia induction or in the post-IR phase significantly increased Arg-II.

Effect of Pio on histopathological changes in renal tissue samples. Sham groups (Sham operation + DMSO, Sham operation + Pio) showed normal histological features of renal parenchyma (both medullary and cortical components) with intact renal corpuscles and tubular segments with almost intact tubular epithelium as well as intact vasculature (Fig. 2A and B; Table II). Renal IRI + DMSO group showed notable degenerative alterations within the epithelium of tubules with moderate dilatation in different segments,

Table I. iNOS, Arginase II and proinflammatory cytokines levels in renal tissue.

Parameter	Sham + DMSO (n=10)	Sham + Pio (n=10)	IRI + DMSO (n=10)	IRI + Pio pre (n=10)	IRI + Pio post (n=10)
iNOS, pg/mg protein	3.38±1.03	2.75±0.68	15.71±2.36[a,b]	6.38±1.54[a-c]	4.33±1.20[c,d]
Arginase-II, pg/mg protein	3.12±0.76	3.40±0.70	2.32±0.74[a,b]	3.04±0.67[c]	3.66±0.65[c]
IL-6, pg/mg protein	5.31±1.28	4.65±0.64	8.05±1.69[a,b]	6.69±0.72[b,c]	5.96±0.92[c]
IL-1 β, pg/mg protein	5.79±0.90	3.90±0.81	18.59±3.55[a,b]	13.76±1.80[a-c]	6.75±1.23[b-d]
TNF-α, pg/mg protein	10.77±1.22	9.12±1.84	55.69±15.09[a,b]	16.43±1.95[c]	11.82±1.52[c]
TGF-β, pg/mg protein	16.48±1.17	16.20±1.45	42.54±7.87[a,b]	21.84±2.02[a-c]	17.50±1.82[c]

$P<0.05$ vs. [a]Sham + DMSO, [b]Sham + Pio, [c]IRI + DMSO and [d]IRI + Pio pre. IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative; iNOS, inducible nitric oxide synthase.
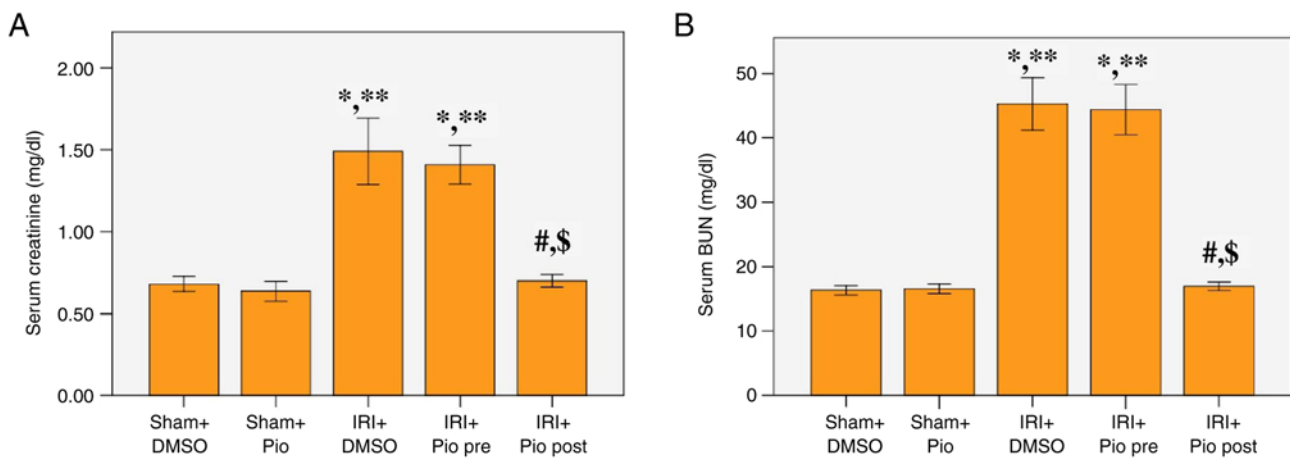

Figure 1. Effect of Pioglitazone on the serum levels of creatinine and BUN. Serum (A) creatinine and (B) BUN at 24 h after renal IRI. *$P<0.05$ vs. Sham + DMSO group, **$P<0.05$ vs.Sham + Pio group, #$P<0.05$ vs. IRI + DMSO, $$P<0.05$ vs. IRI + prophylactic preoperative (pre) Pio. Data shown are means ± SD. BUN, blood urea nitrogen; IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative.

occasional focal records of tubular necrosis with intraluminal casts, congested glomerular tufts and interstitial blood vessels (BVs) and mild inflammatory cell infiltrate (Fig. 2C). Pio administration prior to ischemia induction caused only a mild focal improvement of renal tissue architecture without notable protective efficacy (Fig. 2D), while Pio was effective at improving renal tissue architecture post-IR, as shown by the organized morphological features of renal parenchyma, notable protective efficacy on renal tubular epithelium, mild focal records of degenerated TECs, occasional nuclear pyknosis, mild congested interstitial BVs and glomerular tufts (Fig. 2E).

Effect of Pio on the expression of CD86 in renal tissue. IRI was a potent inducer for CD86 immunoexpression (Fig. 3). A significant increase in the mean area % of CD86 immunoexpression was detected in the IRI + DMSO, IRI + prophylactic preoperative (pre) Pio, IRI + post Pio groups (14.68, 6.58 and 3.95% respectively) compared with the Sham groups (Sham + DMSO, 0.67%; Sham + Pio, 0.52%). Pio, whether administered prior to ischemia induction or in the post-IR phase, caused a significant decrease in CD86 immunoexpression compared with the IRI + DMSO group. Moreover, the decrease in

immunoexpression was more significant when Pio was administered in the post-IR phase.

Effect of Pio on expression of STAT3. Western blotting was performed to detect STAT3 expression levels. The renal IRI + DMSO group showed a significant increase in STAT3 expression compared with Sham groups (Sham operation + DMSO, Sham operation + Pio). Pio administration prior to ischemia did not cause a significant decrease in STAT3 compared with renal IRI + DMSO group, while its administration in the post-IR phase caused a significant decrease in STAT3 compared with the IRI + DMSO group (Fig. 4A and B).

Effect of Pio on expression of miRNA-124 in renal tissue. miR-124 was significantly downregulated in renal tissue of IRI + DMSO group and the group administered Pio prior to ischemia compared with Sham groups (Sham operation + DMSO, Sham operation + Pio). Pio administration prior to ischemia caused a mild increase in miR-124 levels but was not significantly different compared with the IRI + DMSO. A marked increase in miR-124 expression was detected when Pio was administered in post-IR phase compared with IRI + DMSO and IRI + prophylactic preoperative (pre) Pio (Fig. 5).

Table II. Histopathological scoring of renal tissue samples.

Histopathological changes	Sham + DMSO	Sham + Pio	IRI + DMSO	IRI + Pio pre	IRI + Pio post
Tubular degenerative changes	-	-	++++	++++	++
Tubular necrosis	-	-	++	+	-
Congested BVs	-	-	+++	++	++
Inflammatory cell infiltrates	-	-	+	-	-

-, no abnormal cellularity; +, minor focal lesions in 1-3 samples; ++, mild focal lesions in 4-6 samples; +++, moderate diffuse lesions in 4-6 samples and ++++, severe diffuse lesions in all samples; IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative; BV, blood vessel.
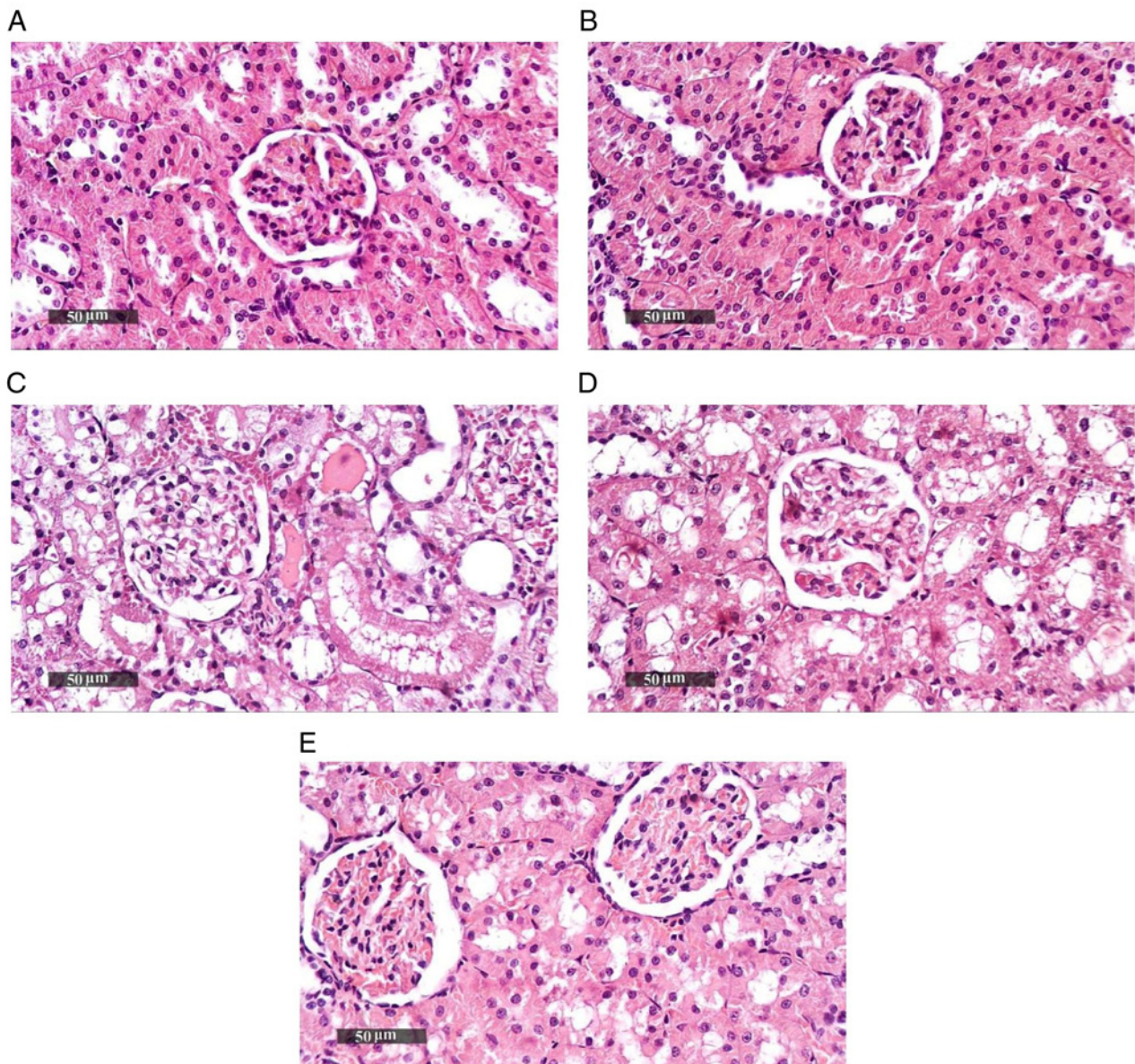

Figure 2. Photomicrographs of hematoxylin and eosin-stained renal tissue sections. (A) Sham + DMSO shows normal histological features of cortical and medullary components of renal parenchyma with apparently intact renal corpuscles and tubular segments with almost intact tubular epithelium as well as intact vasculature. (B) Sham + Pio shows almost the same records as Sham + DMSO without abnormal histological changes. (C) IRI + DMSO shows severe degenerative changes of tubular epithelium with moderate dilatation in different segments, occasional focal records of tubular necrosis with intraluminal casts, severe congested glomerular tufts, congested interstitial BVs and mild inflammatory cell infiltrate. (D) IRI + Pio pre shows almost the same records as IRI group without notable protective efficacy and mild focal improvement of renal tissue architecture. (E) IRI + Pio post shows more organized renal parenchyma with notable protective efficacy on renal tubular epithelium, mild focal records of degenerated tubular epithelial cells, occasional nuclear pyknosis and mild congested interstitial BVs and glomerular tufts. Magnification, x400. IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative; BVs, blood vessels.
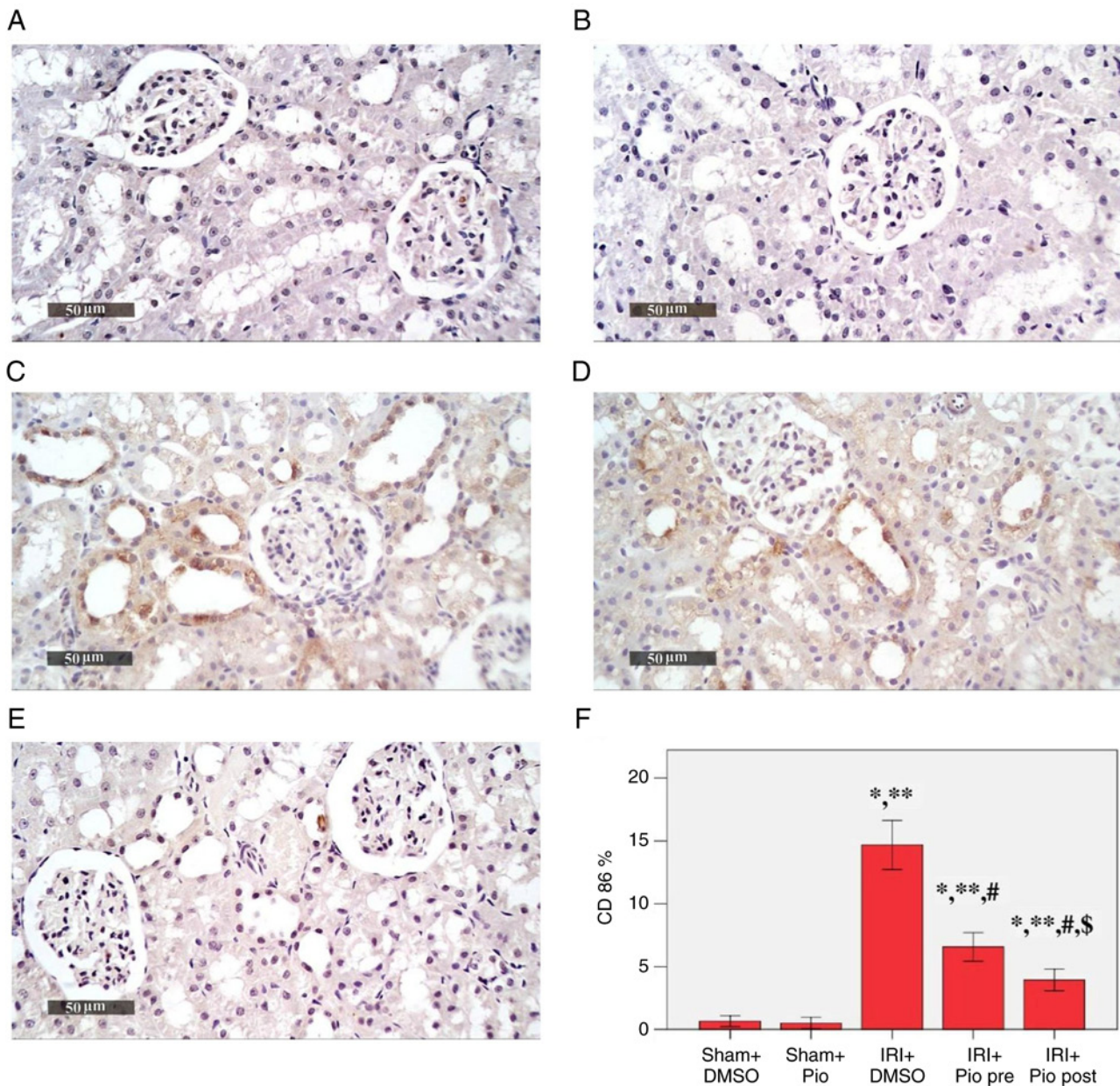
Figure 3. Immunohistochemical detection of CD86 in renal tissue. (A) Sham + DMSO. (B) Sham + Pio. (C) IRI + DMSO (D) IRI + prophylactic preoperative (pre) Pio (E) IRI + postoperative (post) Pio. Magnification, x400. (F) Mean area % of CD86 immunoexpression. Data are expressed as the mean ± SD (n=6). [*]$P<0.05$ vs. Sham + DMSO; [**]$P<0.05$ vs. Sham + Pio; [#]$P<0.05$ vs. IRI + DMSO; [$]$P<0.05$ vs. IRI + prophylactic preoperative (pre) Pio. IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative.

Discussion

Inflammation serves a key role in the pathophysiology and development of renal ischemia-induced AKI (5,40). The tubulointerstitium and renal tubules, which are key sites that respond to injury, comprise a notable part of the kidney. Injured TECs directly (via autocrine function) or indirectly (infiltrating leukocytes via a paracrine process) increase production of inflammatory cytokines (41). TECs are considered key fibrogenic and inflammatory cells (42).

A medication which has been found to have protective functions against renal IRI mouse models is Pio, a synthetic ligand of PPAR-γ. The majority of studies has investigated the renoprotective effect of Pio in renal IR rat models with Pio administered prior to renal ischemia induction (35,43,44). The

present study assessed the ability of Pio to provide protection prior to renal ischemia induction as well as in the post-IR to demonstrate the potential for acute use in AKI.

Here, Pio administration prior to ischemia or in the post-IR phase significantly decreased levels of TNF-α, IL-1β, IL-6, TGF-β and iNOS in renal tissue. Studies have found that PPARγ agonists inhibit inflammation by stopping inflammatory factor synthesis and signaling pathways (45,46).

Notably, two markers of inflammation, iNOS and IL-1β, were significantly decreased in the group administered Pio in the post-IR phase demonstrating the specific differential action of Pio and further supports the findings of previous studies demonstrated the specific effect of PPARγ on iNOS expression and IL-1β levels (47-49). According to Crosby *et al* (50),
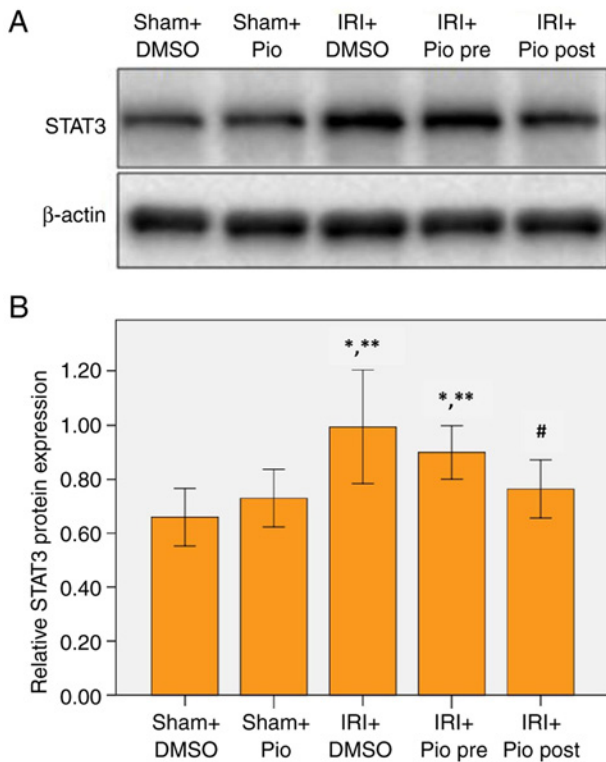
Figure 4. Detection of STAT3 expression. (A) Western blotting for detection of STAT3 protein expression. (B) STAT3/β-actin ratio. *P<0.05 vs. Sham + DMSO; **P<0.05 vs. Sham + Pio; #P<0.05 vs. IRI + DMSO. Data are presented as the mean ± SD. IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative.
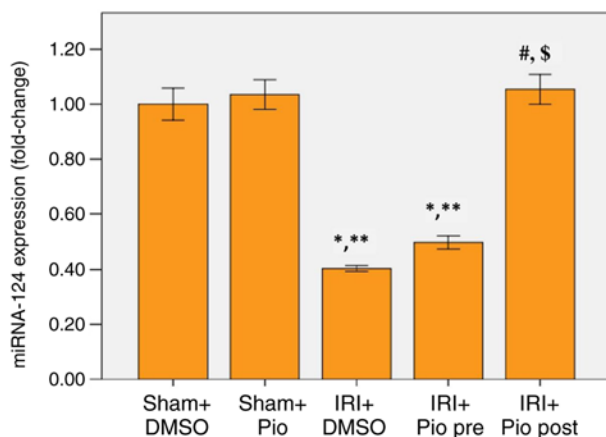

Figure 5. miRNA-124 expression in renal tissue (n=10/group). *P<0.05 vs. Sham + DMSO; **P<0.05 vs. Sham + Pio; #P<0.05 vs. IRI + DMSO; $P<0.05 vs. IRI + prophylactic preoperative (pre) Pio. Data are presented as the mean ± SD. IRI, ischemia reperfusion injury; Pio, pioglitazone; pre, preoperative; post, postoperative; miRNA, microRNA.

in mesangial cells, PPARγ agonists directly inhibit iNOS transcription as well as NO production. PPARγ is a negative regulator of NLRP3 inflammasome activation. PPARγ binding sites are located in the promoter regions of a member of the NLRP3 family, which decrease downstream molecules (such as IL-1β). Activating the NLRP3 inflammasome is associated with renal injury and inflammation in cases of I/R-induced AKI (51-54).

One of the most important reno-protective mechanisms of PPARγ agonists is mediated by inhibitory action on iNOS, as NO generated by iNOS contributes notably to renal IRI. NO reacts with superoxide anion to form peroxynitrite. Peroxynirtrite induces injury by direct oxidant injury and protein tyrosine nitration (55). Furthermore, several studies have reported that inactivation of iNOS expression and activity ameliorates NO-mediated renal injury (11,56,57).

The results of the present study showed significantly decreased levels of Arg-II in the IRI + DMSO group, while Pio increased Arg-II levels. Inhibitory effects of PPARγ agonists on iNOS expression increase the concentration of arginine, a substance used by both Arg and NOS enzymes, resulting in the stimulation of Arg expression (58). Erbas et al (59) reported that the inhibitory effects of N-Acetylcysteine on iNOS activity increased arginine availability, which caused an increase in Arg activity.

In general, the observed decrease in pro-inflammatory cytokines as well as iNOS in the group administered Pio in the post-IR phase compared with dosing prior to ischemia may be associated with Pio pharmacokinetics including time at maximum plasma concentration and elimination half-life.

The present histopathological changes demonstrated the reno-protective effects of Pio administration and confirmed that increased expression of iNOS contributed to increased IR- mediated renal tissue injury. The group given Pio in the post-IR phase showed significantly lower iNOS levels with notably decreased histological evidence of IR-mediated renal tissue injury compared with the group given Pio prior to renal ischemia induction.

To assess the role of TECs as drivers of inflammation, kidney tissue was stained for CD86 to investigate whether they served as APCs. There is conflicting data in terms of expression of CD80 and CD86, which are needed for the activation of CD4+ T cells, in renal epithelium (60,61). The results of the study showed that IRI was a potent inducer for CD86 expression in TECs. Breda et al (62) observed high expression of CD86 in proximal tubular epithelial cells and suggested an inflammation-dependent regulation of epithelium-expressed CD80 and CD86. Niemann-Masanek et al (63) reported that, in addition to generating pro-inflammatory cytokines and chemokines, tubular cells also express complement and their receptors, toll-like receptors, and co-stimulatory molecules (such as CD80 and CD86) which interact with CD28 on T lymphocytes to facilitate production of cytokines.

The present results revealed that Pio administration significantly suppressed the expression of CD86. This raises the question of which mechanism underlies the inhibitory effect of PPARγ agonists on CD86 expression in tubular epithelial cells.

To understand the molecular mechanisms in IRI, expression of STAT3 was assessed in renal tissue as its dysregulated activation is implicated in various types of kidney disease. Here, STAT3 expression was significantly increased in IRI + DMSO group and Pio administration in the post-IR phase significantly decreased STAT3 expression. Evidence suggests therapeutic potential for STAT3 inhibition in numerous pathological renal models, but results of STAT3 inhibition role in AKI is contradictory (24,26). To

clarify the mechanism by which PPARγ agonists suppresses expression of STAT3, the present study assessed the levels of miRNA-124 expression in kidney tissue as it negatively regulates inflammation by targeting several pathways. Previous studies have reported that miRNA-124 targets STAT3 3' untranslated region and inhibits protein translation (32,64-67). The present study showed a significant downregulation of miRNA-124 in the IRI + DMSO group. Pio administered in the post-IR phase significantly upregulated miRNA-124 expression, which explains the significant decrease in STAT3 expression observed in this group. These findings support those of Wang *et al* (31) who demonstrated that activation of PPARγ upregulates miRNA-124 and inhibits miRNA-124 target genes.

To conclude, the present study demonstrated that tubular epithelium serves an important role in the inflammatory response in kidney IRI, not only generating proinflammatory cytokines which activate inflammatory cells, but also expressing CD86, which is required for T lymphocyte activity regulation. Targeting STAT3 by enhancing expression of miRNA-124 may exert beneficial anti-inflammatory effects in kidney IRI. The molecular mechanism by which Pio exerted its anti-inflammatory effect includes upregulation of miRNA-124 with subsequent inhibition of STAT3 expression. Better understanding of the molecular aspects underlying the inflammatory component in kidney IRI may provide novel therapeutic strategies to attenuate inflammation.

Availability of data and materials

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

Authors' contributions

WBEG conceived the study, designed and performed the experiments and wrote and edited the manuscript. MMA conceived the study, designed and performed the experiments and edited the manuscript. SAS wrote the manuscript and contributed to analysis and interpretation of the data. LAM and HEN designed and performed the experiments and wrote the manuscript. AMS performed the histological examination of the kidney and wrote the manuscript. WBEG and MMA confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee, Benha Faculty of Medicine, Benha University, Egypt (approval no. RC.11.6. 2022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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