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# Remote Ischemic Preconditioning Ameliorates Renal Fibrosis After Ischemia-Reperfusion Injury via Transforming Growth Factor beta1 (TGF- $\beta$ 1) Signalling Pathway in Rats

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**Background:** The present study was conducted to explore the influence of remote ischemic preconditioning (RIPC) on the adjustment of renal fibrosis after ischemia-reperfusion injury (IRI).

**Material/Methods:** Male Sprague-Dawley rats were randomly assigned to 3 groups following right-side nephrectomy: the Sham group (without renal artery clamping), the IRI group (45 min left renal artery clamping), and the RIPC group (rats were treated daily with 3 cycles of 5 min of limb ischemia and 5 min of reperfusion on 3 consecutive days before left renal artery occlusion). After 3 months of reperfusion, the renal function and the extent of tubular injury and renal fibrosis were assessed. The expressions of transforming growth factor beta1 (TGF- $\beta$ 1), p-Smad2, Smad2, p-Smad3, and Smad3 were also evaluated.

**Results:** There was no significant difference in renal function and tubular damage among the 3 groups after 45 min of kidney ischemia followed by 3 months of reperfusion. However, an obvious increase of extracellular matrix components and  $\alpha$ -SMA could be observed in the kidney tissues of the IRI group, and the changes were significantly ameliorated in rats treated with enhanced RIPC. Compared with the IRI group, the expression of TGF- $\beta$ 1 and the level of p-Smad2 and p-Smad3 were decreased after the intervention of enhanced RIPC.

**Conclusions:** Enhanced RIPC ameliorated renal fibrosis after IRI in rats, which appears to be associated with inhibition of the TGF- $\beta$ 1/p-Smad2/3 signalling pathway.

**MeSH Keywords:** **Fibrosis • Ischemic Preconditioning • Reperfusion Injury • Transforming Growth Factor beta1**

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/919185>



## Background

Renal ischemia-reperfusion injury (IRI) is an inevitable pathology in various clinical situations, including kidney transplantation, nephron-sparing surgery, renal artery angioplasty, and cardiovascular surgery [1,2]. IRI-induced acute kidney injury (AKI) is one of the main causes of renal fibrosis, characterized by glomerulosclerosis and tubulointerstitial fibrosis, which can drive kidney diseases to the end stage [3,4]. The progressive loss of renal function is often irreversible and is related to high risk of mortality [5]. At present, there is no reliable clinical approach to effectively reverse renal fibrosis, and identifying effective strategy is urgently needed.

Remote ischemic preconditioning (RIPC) is a promising non-pharmacological approach that can protect an organ or tissue by inducing several cycles of transient nonlethal ischemia and reperfusion in another remote organ or issue [6]. Initially, RIPC was introduced to attenuate myocardial IRI and was proved to be helpful [7] and subsequently extended to other vital organs, including the kidneys. Although the exact mechanisms of RIPC remains undefined, the renal-protective role of RIPC against IRI-induced AKI has been validated in clinical settings and animal models [8,9].

Transforming growth factor beta1 (TGF- $\beta$ 1) is one of the most critical factors regulating renal fibrosis [10]. TGF- $\beta$ 1 exerts its biological effects on progression of renal fibrosis through phosphorylation and activation of its downstream Smad signalling pathway [11]. It has been confirmed that RIPC can alleviate renal IRI by downregulation of TGF- $\beta$ 1 [12]. However, previous studies have mainly focused on the effects of RIPC on short-term renal function after IRI, with little attention to long-term renal fibrosis.

We thus conducted the present study to investigate the effects of RIPC on renal fibrosis after kidney ischemia-reperfusion in a rat model, and assessed the role of the TGF- $\beta$ 1/Smad signalling pathway in this process.

## Material and Methods

### Animals

We used male Sprague-Dawley rats, age 8–12 weeks and weighing 250–300 g, which were housed in the animal house of the Experimental Animal Center affiliated with Nanjing First Hospital. All the animals were fed in separate cages with stable temperature and humidity, on a 12-h light/dark cycle, and provided free access to food and water. All animal operations and treatments were approved by the Institutional Animal Care and Use Committee of Nanjing First Hospital, Nanjing

Medical University. This study was conducted in accordance with institutional and national guidelines for treatment of laboratory animals.

### Experiment design

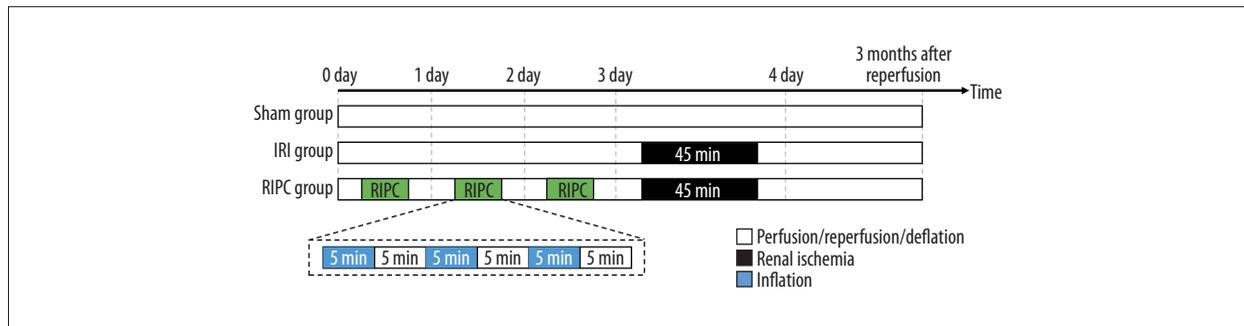
All rats were anaesthetized by sodium pentobarbital injection (50 mg/kg, i.p.), and a warming table with constant temperature was utilized to maintain body temperature. After right-side nephrectomy, the rats were randomly assigned to 3 groups (12 in each group): a Sham group in which the left side renal artery was separated but not clamped; an IRI group in which the left renal artery was separated and occluded for 45 min using a non-traumatic vascular clamp; and an RIPC group in which the left hind limb was treated daily with 3 cycles of 5 min each of ischemia and 5 min of reperfusion for 3 consecutive days before occlusion of the left renal artery (Figure 1). The RIPC procedure was implemented through the inflation (inflated to 250 mmHg) and deflation of a cuff around the left hind limb, with the absence of femoral pulse indicating successful occlusion of the hind limb bloodstream. All animals were anesthetized again to collect blood samples and to harvest left kidney tissues at 3 months after reperfusion.

### Renal function analysis

Blood samples were taken from the inferior vena cava and centrifuged at 3000 rpm for 10 min. Supernatants from each sample were subsequently collected to assess serum levels of blood urea nitrogen (BUN) and serum creatinine (SCr) by using clinically automated analysis methods (Hitachi 7600-10; Hitachi High-Technologies Corp.).

### Histological examination

The kidney samples collected at 3 months after reperfusion were fixed with 10% formaldehyde, then gradually dehydrated and embedded in paraffin. The paraffin-embedded samples were sectioned at 5  $\mu$ m and then stained with hematoxylin and eosin (H&E), Sirius red, and Masson's trichrome. An experienced pathologist who was not aware of the experimental plan assessed the stained slides using a standard light microscope (Olympus BX53). As described previously [13], the degree of tubular injury was quantitatively assessed with a scoring system based on pathological changes, including tubular dilatation, cast formation, tubular necrosis, and vacuolization, ranging from Grade 0 to 5 (0, normal kidney; 1,  $\leq$ 10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; 5,  $\geq$ 76%). Renal fibrosis was histologically evaluated in slides stained with Masson's trichrome and Sirius red using Image Pro Plus (Media Cybernetics, Inc., Version 6.0.0.260), and are presented as a percentage of the total area. The sections in the present study were all observed at 400 $\times$  or 200 $\times$  magnification, and all microscopic assessments



**Figure 1.** Schematic illustration of the experimental protocols. IRI – ischemia-reperfusion injury; RIPC – remote ischemic preconditioning.

of the sections were performed in a randomized manner (20 random fields from each section evaluated at 400× magnification and 10 random fields from each section evaluated at 200× magnification).

### Immunohistochemical analysis

Tissue sections (5 μm) were made as described above. After deparaffinizing, rehydrating, antigen retrieval, and blocking, the sections were incubated overnight at 4°C with rabbit anti-collagen I antibody (Abcam), rabbit anti-collagen III antibody (Abcam), rabbit anti-fibronectin antibody (Abcam), and rabbit anti-alpha smooth muscle actin (α-SMA) antibody (Abcam), respectively. On the next day, samples were washed with phosphate-buffered saline (PBS) 3 times for 5 min each time, before incubation with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (KeyGEN Biotechnology) for 30 min. Diaminobenzidine (DAB) (KeyGEN Biotechnology) was used for coloring. Immunohistochemical assay of these outcomes was performed using Image Pro Plus software.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from kidney tissues by use of TRIzol® reagent (Life Technology). The concentration of extracted RNA was determined by spectrophotometry (BioPhotometer; Eppendorf, Germany), and its purity was assessed by the absorbance ratio of 260 nm/280 nm. Total isolated RNA was reverse-transcribed into cDNA using oligo dT primers and Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time PCR was performed on the 7300 Sequence Detection System using a SYBR Green-based real-time detection method [Roche, Mannheim]. The primer sequences were:  
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-TGTCGTGGAGTCTACTGGCGTCTT-3', reverse 5'-GAGGGAGTTGTCATATTCTCGTGGT-3';  
TGF-β1, forward 5'-ACGTCAGACATTCGGAAGCAGTG-3', reverse 5'-GCAAGGACCTGCTGACTGTGTG-3'.

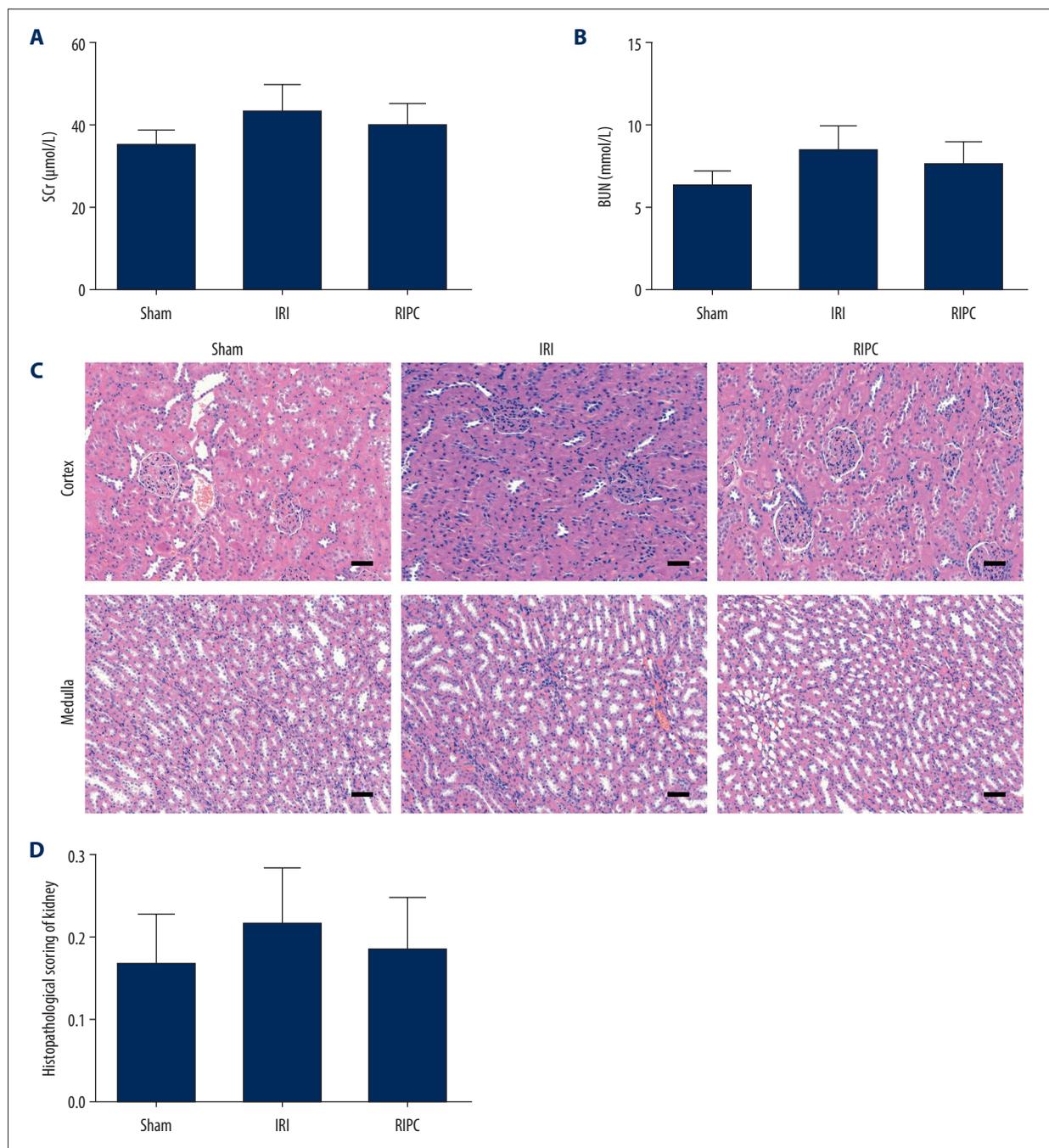
To calculate the relative expression level of the products, the  $2^{-\Delta\Delta CT}$  method was used and results were normalized to GAPDH.

### Western blotting analysis

The kidney samples of all rats were homogenized, and proteins were extracted using an extraction kit (KeyGEN Biotechnology). The concentration of protein was quantified using a Pierce BCA protein assay kit (Beyotime) in accordance with the manufacturer's instructions. Equivalent amounts of proteins were added to each lane and subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the detached proteins were relocated to the membranes of polyvinylidene difluoride (PVDF) (Millipore). After blocking with 5% non-fat dry milk in Tris-buffered saline mixed with 0.1% Tween-20, the membranes were incubated all night at 4°C with corresponding primary antibodies: collagen I (1: 1000, Abcam), collagen III (1: 1000, Abcam), fibronectin (1: 1000, Abcam), α-SMA (1: 1000, Abcam), TGF-β1 (1: 2000, Abcam), Smad2 (1: 1000, Bioss), phospho-Smad2 (p-Smad2, 1: 500, Abcam), Smad3 (1: 1000, Bioss), and phospho-Smad3 (p-Smad3, 1: 1000, Abcam), followed by 2 h of continuous incubation with secondary antibody conjugated with horseradish peroxidase (KeyGEN Biotechnology) at room temperature. The bands were detected by Pierce ECL Western blotting substrate (Life Technologies). GAPDH was treated as an internal control. The final data were semi-quantified using Image J software (NIH, MD).

### Statistical analysis

All data are presented as mean±SEM and analyzed using SPSS version 22.0 (IBM SPSS Statistics). One-way analysis of variance (ANOVA) was performed to assess differences among groups. If ANOVA revealed a significant difference, the post hoc Tukey test was performed for pairwise comparison among groups. The level of statistical significance for all analyses was set at  $p < 0.05$ .



**Figure 2.** Renal function and morphologic features evaluated by H&E staining at 3 months following reperfusion. **(A)** Serum creatinine (SCr,  $\mu\text{mol/L}$ ); **(B)** Blood urine nitrogen (BUN,  $\text{mmol/L}$ ); **(C)** Representative images of H&E staining in the cortex and medulla of kidney tissues; **(D)** Tubular injury score in the 3 groups. Scale bars=50  $\mu\text{m}$ .

## Results

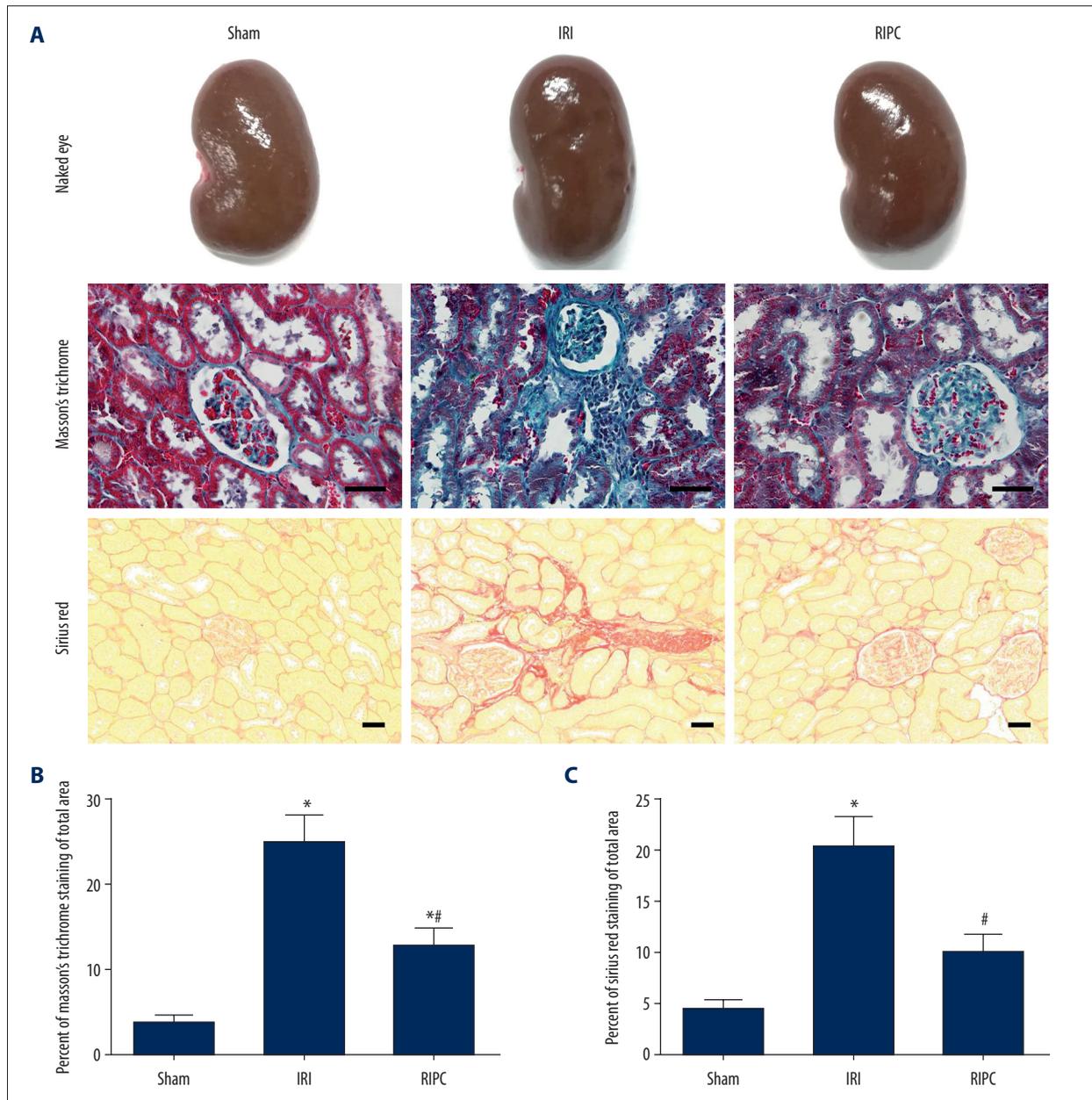
### Examination of renal function

After 45 min of kidney ischemia 3 months after reperfusion, SCr and BUN were slightly increased in the IRI and RIPC group,

but there were no significant differences among the 3 groups (Figure 2A, 2B).

### Renal morphology features

As demonstrated in Figure 2C, recognized features of severe tubular injury, characterized by cast formation, vacuolization,

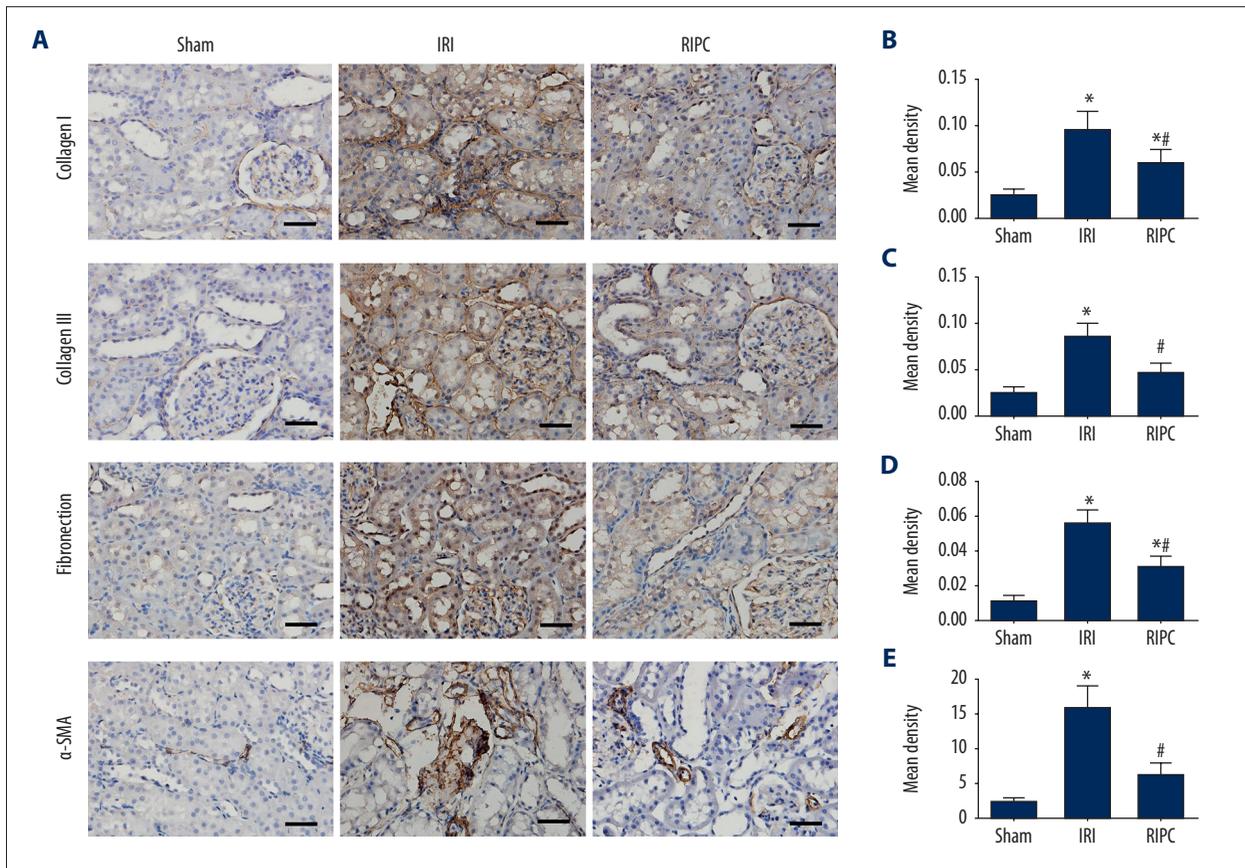


**Figure 3.** Unmagnified view with Masson's trichrome and Sirius red staining for assessing renal fibrosis at 3 months after reperfusion. (A) The appearance of the kidneys and representative sections stained with Masson's trichrome and Sirius red in the 3 groups; (B) Semi-quantitative analysis of Masson's trichrome staining; (C) Semi-quantitative analysis of Sirius red staining. Scale bars=50 µm. \*  $p < 0.05$  vs. Sham group; #  $p < 0.05$  vs. IRI group.

tubular dilatation, and necrosis, were rarely detected in the cortex and medulla among the 3 groups. The histopathological scoring was used to quantify tubule damage, which also revealed no significant differences among the 3 groups (Figure 2D). Furthermore, increased cell infiltration and extracellular matrix (ECM) component within the renal interstitium were observed in the IRI and RIPC groups (Figure 2C).

### Effects of RIPC on renal fibrosis

As shown in Figure 3A, at 3 months after reperfusion, the appearance of harvested kidneys in the IRI group and RIPC group was uneven, with many obvious depressed scars, while kidneys of the Sham group were smooth. We performed Masson's trichrome and Sirius red staining to semi-quantify the degree of renal fibrosis. Kidney tissues stained with Masson's trichrome in the IRI group revealed a remarkably increased expression of collagen in



**Figure 4.** Expression of collagen I, collagen III, fibronectin, and  $\alpha$ -SMA assessed by immunohistochemistry in the renal tissues at 3 months following reperfusion. **(A)** Representative images of immunohistochemistry staining; **(B–E)** Semi-quantitative evaluation of sections stained with collagen I, collagen III, fibronectin, and  $\alpha$ -SMA, respectively. Scale bars=50  $\mu$ m. \*  $p < 0.05$  vs. Sham group; #  $p < 0.05$  vs. IRI group.

interstitium compared to that in Sham kidneys (Figure 3A, 3B). In contrast, RIPC treatment significantly decreased the collagen content in interstitium after acute renal IRI (Figure 3A, 3B). The results of Sirius red staining demonstrated a similar trend of collagen expression among the 3 groups (Figure 3A, 3C).

We also used immunohistochemistry and Western blot analysis to detect the expression of collagen I, collagen III, fibronectin, and  $\alpha$ -SMA in renal tissues. Results from these 2 methods further confirmed a significant accumulation of collagen I, collagen III, fibronectin, and  $\alpha$ -SMA in kidneys of the IRI group 3 months after reperfusion when compared with the Sham group (Figures 4, 5). All of the accumulation of relevant ECM components and  $\alpha$ -SMA in kidney tissues were significantly reduced in rats treated with RIPC (Figures 4, 5).

#### Effects of RIPC on TGF- $\beta$ 1, p-Smad2 and p-Smad3 expression in kidney tissues

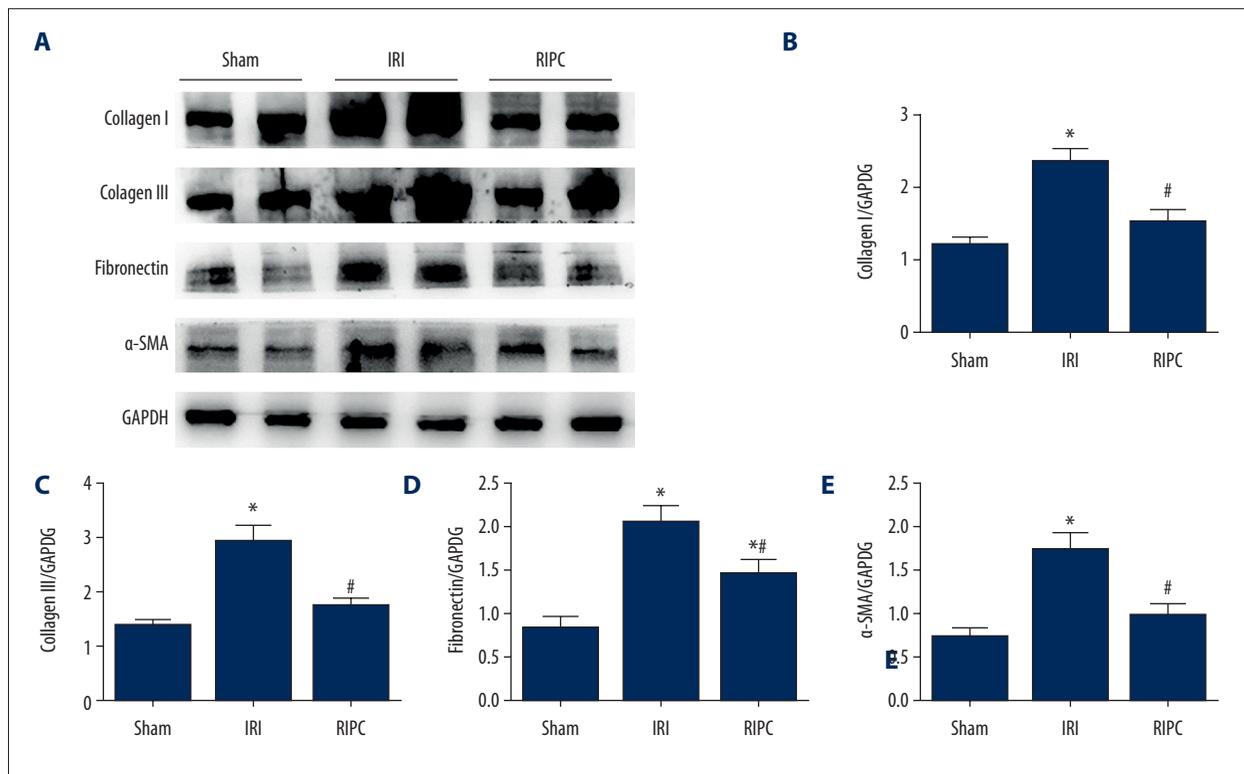
TGF- $\beta$ 1 expression was remarkably elevated in harvested kidneys of the IRI group at 3 months after acute IRI compared

with the Sham group, as assessed by Western blotting and qRT-PCR (Figure 6A–6C). After treatment with RIPC, the increase of TGF- $\beta$ 1 protein level and mRNA expression were significantly inhibited (Figure 6A–6C).

Moreover, there was no obvious difference in the level of Smad2 among the 3 groups (Figure 6D). However, the level of p-Smad2 and the ratio of p-Smad2 to Smad2 in kidney tissues were significantly heightened in the IRI group compared to the Sham group (Figure 6D–6F). These changes were obviously attenuated in kidneys of rats treated with RIPC (Figure 6D–6F). The same tendency was detected in the level of p-Smad3 (Figure 6D, 6G, 6H).

## Discussion

Renal fibrosis is a common and severe complication in patients with IRI-induced AKI [3–5]. Numerous clinical and pre-clinical studies have demonstrated that RIPC can alleviate kidney injury at the acute stage after ischemia-reperfusion [6,8,9,14–16],



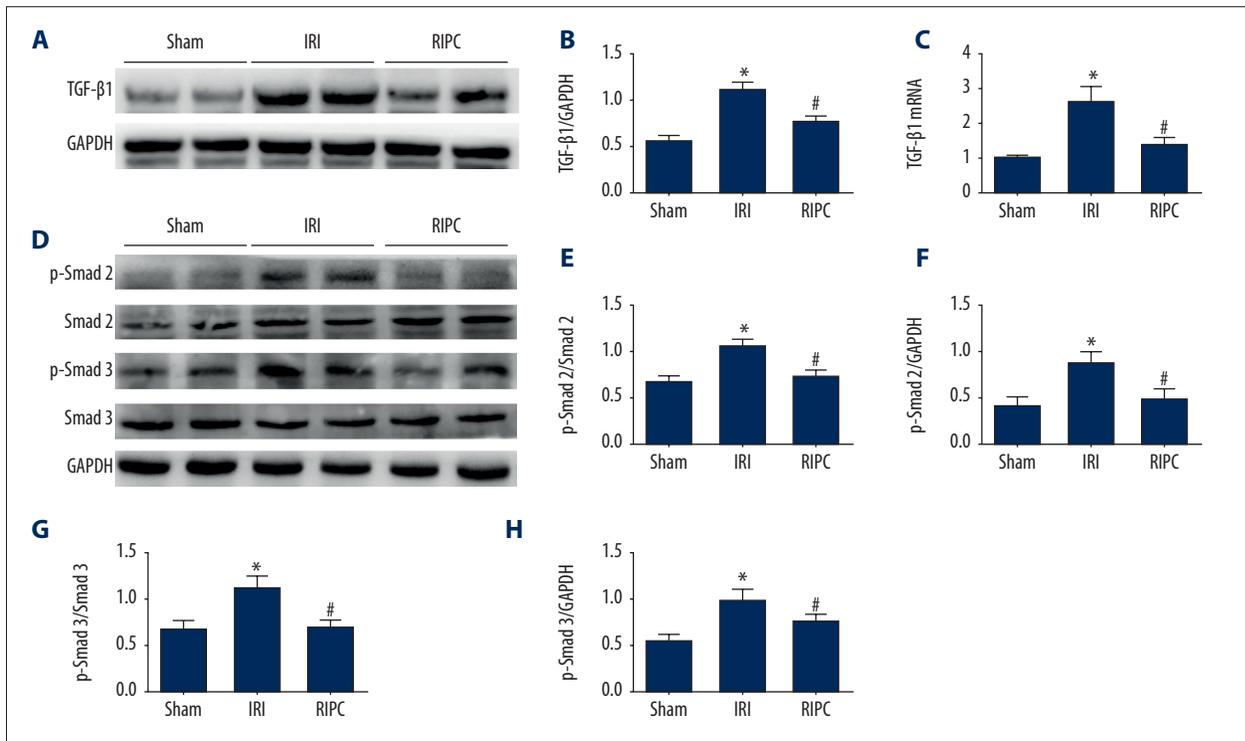
**Figure 5.** Expression of collagen I, collagen III, fibronectin, and  $\alpha$ -SMA assessed by Western blotting in the kidneys at 3 months following reperfusion. **(A)** Representative Western blots show the effects of RIPC on collagen I, collagen III, fibronectin, and  $\alpha$ -SMA expression levels; Relative abundance of collagen I/GAPDH **(B)**, collagen III/GAPDH **(C)**, fibronectin/GAPDH **(D)**, and  $\alpha$ -SMA/GAPDH **(E)**. \*  $p < 0.05$  vs. Sham group; #  $p < 0.05$  vs. IRI group.

but its long-term effects on renal fibrosis have received little research attention. To the best of our knowledge, this is the first study demonstrating that RIPC, a safe and non-invasive intervention, induced by 3 daily cycles of limb ischemia and reperfusion on 3 consecutive days before prolonged kidney ischemia, can attenuate renal fibrosis after ischemia-reperfusion injury. Inhibiting the TGF- $\beta$ 1/p-Smad2/3 signalling pathway may play a critical role in this process.

Prolonged ischemia and subsequent reperfusion of the kidney can lead to AKI, characterized by elevated serum markers and tubular damage in many clinical settings, which is invariably an inevitable event [1,2]. In clinical practice, we generally use serum markers to determine the damage and recovery of the kidney after renal or non-renal surgeries. However, evidence from our previous study confirmed that kidney function will gradually recover within 1 week after renal ischemia-reperfusion, even without any interventions [17]. Thus, it is necessary to pay attention to the long-term impact of interventions on the kidneys after ischemia-reperfusion, rather than merely focus on the short-term effects. The results of our study showed that the renal function and tubular damage were mostly restored in both RIPC-treated and -untreated groups at 3 months after reperfusion, and there were no significant

differences compared with the Sham group. When we focused on the results of renal fibrosis, the differences among the 3 groups became apparent. Although the kidney damage was relatively mild, the accumulation of relevant ECM components in kidney tissues was significantly increased in rats of the IRI group compared to the Sham group, and RIPC remarkably decreased the accumulation.

RIPC was initially reported by Przyklen et al., and proven to be effective in the protection of myocardium from lethal IRI induced by sustained coronary occlusion [7]. As a technology similar to RIPC, ischemic preconditioning (IPC), defined as brief nonlethal episodes of ischemia and subsequent reperfusion in a target organ, is also used to prevent lethal IRI [18]. Compared to IPC, which lengthens the total ischemic time of the kidney, RIPC initiated in the limb or other remote organs must be safer and more applicable in clinical settings, especially in some non-renal operations. RIPC is usually induced by a single session of repeated cycles of ischemia and reperfusion immediately before prolonged kidney ischemia [6,14], but the renal-protective role of this type of RIPC remains unclear. Evidence from multiple clinical and pre-clinical studies demonstrated that late RIPC induced 24 h before prolonged kidney ischemia provides more powerful renal protection than the traditional



**Figure 6.** Expression of TGF- $\beta$ 1, p-Smad2, Smad2, p-Smad3, and Smad3 in the kidneys at 3 months after reperfusion. (A, D) Representative Western blots show the effects of RIPC on TGF- $\beta$ 1, p-Smad2, Smad2, p-Smad3, and Smad3 expression levels; (C) Effects of RIPC on TGF- $\beta$ 1 mRNA expression; (B, E-H) Relative abundance of TGF- $\beta$ 1/GAPDH, p-Smad2/Smad2, p-Smad2/GAPDH, p-Smad3/Smad3 and p-Smad3/GAPDH, respectively. \*  $p < 0.05$  vs. Sham group; #  $p < 0.05$  vs. IRI group.

short-window RIPC [19–21]. Jones et al. also revealed that daily exposure of the limb to RIPC rather than a single dose can improve local and systemic endothelial function and microcirculation in humans [22], which was also verified by other research [23,24]. Considering the optimal efficacy of RIPC and limited time frame before surgery, we adopted a new strategy to induce an enhanced RIPC, in which the intermittent limb ischemia was performed once daily on 3 consecutive days before kidney ischemia. The results of the present study revealed that the enhanced RIPC also significantly attenuated renal fibrosis, as shown by the recovery of renal function.

Progressive renal fibrosis is a complex pathophysiological process generally mediated by multiple mediators, including growth factors, cytokines, metabolic toxins, and stress molecules, and corresponding multiple pathways [25]. Among them, TGF- $\beta$ 1/Smad signalling is widely recognized as an essential target in the pathogenesis of renal fibrosis due to its role in increasing ECM components produced from fibrogenic cells and inducing the transformation of tubular epithelial cells to myofibroblasts through epithelial-mesenchymal transition (EMT) [25–27]. Results from the present study revealed that the protein level and mRNA expression of TGF- $\beta$ 1 in kidney tissues were obviously increased after 45 min of kidney ischemia and at 3 months after reperfusion, while RIPC inhibited

the rise in TGF- $\beta$ 1 in the impaired kidneys. We also explored the levels of Smad2 and Smad3 and their corresponding phosphorylated proteins in kidney tissues. The phosphorylation of smad2 and smad3 was significantly increased long after acute renal IRI, and the treatment of RIPC reduced the levels of p-Smad2 and p-Smad3. Meanwhile, the increased ECM components, including collagen I, collagen III, and fibronectin, were remarkably decreased by RIPC intervention. The gain of mesenchymal phenotype  $\alpha$ -SMA in tubular epithelial cells is a critical marker of EMT [28,29]. We further found that the expression of  $\alpha$ -SMA was decreased after inducing RIPC, which indicated that RIPC alleviated EMT in kidneys after acute IRI.

Several limitations of this study should be acknowledged when interpreting the results. Firstly, we merely observed the effects of RIPC on renal fibrosis at 3 months after reperfusion, and the longer-term renoprotective role of RIPC was not assessed. Secondly, unlike clinical settings with many confounding factors, the present study only assessed the efficacy of RIPC in a standardized rat model, and its clinical effects remained to be verified.

## Conclusions

We demonstrated that non-invasive enhanced RIPC protected against renal fibrosis after IRI. This protective effect might be achieved through inhibiting the TGF- $\beta$ 1/p-Smad2/3 signalling pathway. Well-designed clinical trials with large sample sizes are needed to further evaluate the effect of RIPC on renal fibrosis.

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## Conflict of interests

None.