Journal of the Formosan Medical Association (2015) 114, 430–437

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

The administration of erythropoietin attenuates kidney injury induced by ischemia/reperfusion with increased activation of Wnt/β-catenin signaling

Xiao Chen, Cen-Cen Wang, Shu-Min Song, Shi-Yao Wei, Jian-Si Li, Shi-Lei Zhao, Bing Li*

Department of Nephrology, Second Affiliated Hospital, Harbin Medical University, Harbin, China

Received 20 October 2014; received in revised form 9 January 2015; accepted 13 January 2015

KEYWORDS
erythropoietin;
kidney ischemia/reperfusion injury;
micro-RNA;
Wnt/β-catenin

Background/purpose: Understanding the mechanisms of protecting the kidneys from injury is of great importance because there are no effective therapies that promote repair and the kidneys frequently do not repair adequately. Evidence has shown that erythropoietin (EPO) has a vital renoprotective role, independent of its erythropoietic effect. However, whether EPO can contribute to kidney repair after injury and the potential mechanisms are not fully understood.

Methods: To investigate the renoprotective mechanism of EPO, a kidney ischemia/reperfusion injury (IRI) model was induced in adult male Sprague–Dawley rats. The rats were subsequently randomly treated with EPO or a vehicle 6 hours after the kidney IRI. The rats were sacrificed on Day 3, Day 5, and Day 7 post kidney IRI. Renal function and histological alterations were examined. Renal interstitial macrophage infiltration, cell proliferation, apoptosis, and angiogenesis were evaluated by immunostaining. Furthermore, the effects of EPO on the Wnt/β-catenin pathway and IRI-related micro-RNAs were investigated.

Results: The administration of EPO significantly improved renal function and reduced tubular injury. Furthermore, EPO treatment significantly prevented tubular cell apoptosis and promoted cell proliferation after IRI. Erythropoietin significantly suppressed macrophage infiltration, compared to the vehicle. In addition, treatment with EPO markedly prevented the loss of microvasculature. We have also demonstrated that, compared to the vehicle, EPO administration enhanced the expression of Wnt7b and β-catenin, and downregulated miR-21, -214, -210, and -199a.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

* Corresponding author. Department of Nephrology, Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Road, Nangang District, Harbin, 150086, China.
E-mail address: icecreamlee@hotmail.com (B. Li).

http://dx.doi.org/10.1016/j.jfma.2015.01.007

Conclusion: Erythropoietin protects the kidneys against IRI by attenuating injury of the renal microvasculature and tubule epithelial cells, by promoting Wnt/β-catenin pathway activation, and by regulating miRNA expression.

Introduction

Renal ischemia/reperfusion injury (IRI) is a common cause of acute kidney injury (AKI).[1,2] It results from a sudden transient drop in total or regional blood flow to the kidneys.[3] Despite advances in preventative strategies and support measures, this disease is associated with significant morbidity and mortality.[2] Many treatment strategies have been proposed to improve IRI, but few are effective.[3]

Erythropoietin (EPO) is a hematopoietic hormone produced primarily by the adult kidneys.[4] It is a multifunctional cytokine involved in a variety of processes in the kidneys, liver, and brain that are independent of erythropoiesis. Evidence has demonstrated that EPO has cytoprotective effects, which mediate antioxidative stress, antiapoptosis/necrosis, and anti-inflammatory responses,[5,6] and immunomodulation of autoimmune diseases.[7] Erythropoietin may be an efficient renoprotective agent against renal dysfunction and injury. However, the precise mechanisms of EPO involved in kidney IRI have not yet been fully elucidated.

The term "acute tubular necrosis" is used to designate AKI resulting from characteristic pathologic damage to the tubules. The improvement of renal function after kidney IRI primarily depends on the repair and regeneration of injured tubular epithelial cells.[8,9] In particular, IRI causes sterile inflammation, which increases the production of inflammatory cytokines and infiltration of neutrophils and macrophages.[10] Peritubular capillary (PTC) loss is also positively associated with tubular damage in the kidneys in ischemic AKI and in the clinical biopsy.[11,12] Our previous studies also suggested that PTC loss because of ischemia is directly correlated with impaired renal function and the development of renal fibrosis.[13,14] Promoting the repair and regeneration of injured kidney vasculature successfully reverses these pathological changes.[14,15]

Wnt signaling was originally identified as involved in the developmental fate decision and neoplasia. The canonical Wnt pathway can drive cell proliferation and maintain the dedifferentiated state of cells. It also has a critical role in kidney repair and regeneration after IRI.[16] Reparative macrophages may secrete Wnt ligands, which interact with Wnt-expressing receptors on the epithelial cell surface to repair the injured kidney.[13] Micro-RNAs (miRNAs) are endogenous noncoding RNAs that posttranscriptionally regulate gene expression. Research studies have shown that miRNAs are involved in various different biological processes such as hypoxia, differentiation, inflammation, cell proliferation, cell death, and fibrosis in kidney disease,[17,18] and in kidney IRI.[19] Whether EPO can regulate miRNAs in kidney IRI is unknown.

In this study, we aimed to reveal the multifaceted renoprotective roles of EPO in kidney IRI from various respects such as tubular cell apoptosis; macrophage infiltration; the loss of microvasculature; and the expression of Wnts, β-catenins, and IRI-related miRNAs such as miR-21, -199a, -210, and -214. Such efforts may provide better understanding of the renal-friendly benefits of EPO.

Materials and methods

Animals

Male Sprague—Dawley (SD) rats weighing 200—250 g were purchased from the Second Affiliated Hospital of Harbin Medical University Laboratories (Harbin, China). All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All rats were maintained in filter top cages and received sterilized food and acidified water. Experimental protocols were approved by the animal committee of Harbin Medical University (Harbin, China).

Animal model

Male SD rats (200—250 g) were subjected to kidney IRI by methods described previously.[13,14] The warm ischemic time was 45 minutes (i.e., the unilateral model) or 40 minutes (i.e., the bilateral model). All male SD rats were randomly divided into three groups: the sham group, the EPO group, and the vehicle group. Six hours after inducing unilateral IRI, the unilateral model rats intraperitoneally received 2000 IU/kg EPO (EPO group; $n = 24$; rhEPO; Roche, Mannheim, Germany) or received the vehicle (vehicle group; $n = 24$). The sham-operated rats, which underwent the same surgical procedure but without the placement of the vascular clamp, served as controls (i.e., sham group). The rats were sacrificed on Day 3, Day 5, and Day 7 after the operation ($n = 8$ rats per time point).

Six hours after the IRI, the rats with bilateral IRI also received rhEPO (Roche; i.e., EPO group) or the vehicle (i.e., vehicle group). Plasma samples were drawn from the tail vein on Day 1, Day 3, and Day 7 after injury to analyze the creatinine level using previously described methods.[13,14] The bilateral model rats were sacrificed on Day 7 ($n = 8$ rats per group).

Tissue preparation, immunostaining, imaging, and quantification of injury and repair

Rats were perfused with ice cold normal saline, and then preserved in opti-mumcutting temperature compound (OCT) (−80°C) and paraffin by using previously described methods.[13,14] The paraffin sections (3 μm) were processed

and stained with hematoxylin-eosin (HE). Immunofluorescence labeling was performed on 4 μm cryosections. Primary antibodies against the following proteins were used: rabbit anti-rat CD68 (1:200, Abcam; Hong Kong, China); rabbit anti-rat Ki67 (1:200, clone SP6; Thermo Scientific, Fremont, CA, USA); mouse anti-rat RECA-1 (1:20; Abcam, Hong Kong, China), and rabbit anti-rat β-catenin (1:200; Abcam, Hong Kong, China). The secondary antibodies were Alexa Fluor 488-conjugated goat antirabbit; Alexa Fluor 594-conjugated donkey antimouse and Alexa Fluor 594-conjugated goat antirabbit (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

The HE-stained paraffin sections used a blinded scoring method, as reported by our previous research.[14] For each square, the presence of tubule injury (e.g., tubule flattening, necrosis, apoptosis, or the presence of casts) was a positive score. The final score was presented as the percentage of positive squares. Macrophages were identified as positive for CD68 if $>$ 75% of the cell area surrounding the nucleus exhibited Alexa Fluor 488 fluorescence. Specific cells were counted in 10 random cortical interstitial fields per rat. Apoptotic cells were detected by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay kit (Roche, Indianapolis, IN, USA), as previously described.[14] Nuclei were stained using 4,6-diamidino-2-phenylindole. Peritubular capillary (PTC) loss was determined by assessing anti-RECA-1. In brief, images were captured by digital imaging (×200 magnification) sequentially over the entire sagittal section, which incorporated the cortex and outer medulla (10—20 images). Each image was divided into 192 squares by a grid. A square without a PTC was considered positive for loss. Images of β-catenin staining were captured in 10 random medullar fields per rat, and the mean area of positive staining was quantified. Images were obtained by Nikon microscopy (Nikon, Tokyo, Japan) and processed by NIS-Elements software.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was used for the quantitative detection of messenger RNA (mRNA) and micro-RNA expression. TRIzol reagent (Invitrogen, Shanghai, China) was used to extract RNA from cryopreserved renal tissue (−80°C). The RNA was reverse transcribed into cDNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Table 1 shows the valid sequences of the primers. For real-time PCR, 2×SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) was used in accordance with the manufacturer's instructions with the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Singapore, Singapore). After a brief heating of 10 minutes at 95°C, amplification was performed, as follows: 95°C for 15 seconds, 60°C for 1 minute, and 40 cycles. Cycle threshold values of the mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the cycle threshold values of the miRNA were normalized to those of U6. Fold differences in gene expression were determined using the $2^{-\Delta\Delta CT}$ method, and

Table 1 Primers (mouse) used in the experiment.

The studied gene		Sequence of oligonucleotides
Wnt7b	F	CCTTCACATACGCCATCACC
	R	TGCCTGGTTGTAGTAGCCTTG
GAPDH	F	CGCATCTTCTTGTGCAGTG
	R	GAGGGTGCAGCGAACTTTATT
rno-miR-21-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
	F	GCGGCGGTAGCTTATCAGACTG
	R	ATCCAGTGCAGGGTCCGAGG
rno-miR-192-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCTGT
	F	GCGGCGGCTGACCTATGAATTG
	R	ATCCAGTGCAGGGTCCGAGG
rno-miR-24-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGTTC
	F	GCGGCGGTGGCTCAGTTCAGCAG
	R	ATCCAGTGCAGGGTCCGAGG
rno-miR-214-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCCT
	F	GCGGCGGACAGCAGGCACAGAC
	R	ATCCAGTGCAGGGTCCGAGG
rno-miR-210-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCC
	F	GCGGCGGCTGTGCGTGTGACAG
	R	ATCCAGTGCAGGGTCCGAGG
rno-miR-199a-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCA
	F	GCGGCGGACAGTAGTCTGCAC
	R	ATCCAGTGCAGGGTCCGAGG
U6	RT	CGCTTCACGAATTTGCGTGTCAT
	F	GCTTCGGCAGCACATATACTAAAAT
	R	CGCTTCACGAATTTGCGTGTCAT

F = forward; R = reverse; RT = reverse transcription.

were analyzed relative to the standard of the sham group on Day 3.

Statistical analysis

All values are presented as the mean ± the standard deviation. One-way analysis of variance (ANOVA) with the Steel–Dwass test was employed to analyze multiple comparisons. A value of $p < 0.05$ was considered significant in all statistical tests. All tests were conducted using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Results

The administration of EPO improves renal function and attenuates pathological alteration after kidney IRI

Serum creatinine was assessed in the sham surgery rats on Day 0, and then on Day 1, Day 3, and Day 7 after bilateral kidney IRI. Bilateral IRI resulted in a level that was seven times higher (3.91 ± 0.31 mg/dL), compared to the preoperative level (Day 0, 0.52 ± 0.14 mg/dL). By contrast, the level of serum creatinine after EPO treatment was significantly decreased on Day 1, Day 3, and Day 7 after kidney IRI (Fig. 1A). After the IRI, the renal tubules also showed remarkable injury such as tubular swelling and deformation, expansion, inflammatory cell infiltration, tubular epithelial cell degeneration and necrosis, and accumulation of necrotic material within the lumen. Treatment with EPO significantly attenuated these pathological changes (Fig. 1B). These results are expressed graphically in Fig. 1C.

Therapy by EPO prevents cell apoptosis, promotes cell proliferation, and attenuates macrophage infiltration and PTC loss after kidney IRI

To further study the mechanism of reduced renal injury by EPO, we analyzed kidney sections for cellular apoptosis and proliferation. The number of TUNEL+ apoptotic cells increased rapidly in a short time, and gradually decreased with reperfusion time. Treatment with EPO significantly reduced the number of apoptotic cells, as Fig. 2A shows. In addition, the pan cell cycle marker Ki67 was used to label the cell cycle of the kidney cells. The administration of EPO significantly enhanced proliferation, compared to the proliferation in the kidneys of untreated rats on Day 5 and Day 7 after IRI (Fig. 2B).

A significant increase in macrophages was observed in the IRI rats, compared to the number of macrophages in the sham surgery rats. We also observed that EPO could suppress the number of CD68+ macrophages, especially on the 5th day and 7th day after IRI (Fig. 2C). To analyze PTC regeneration, we used RECA-1 as a marker of endothelial cells to evaluate PTC loss. Peritubular capillary loss increased in kidney tissues with IRI, but significantly improved after EPO treatment on Day 3, Day 5, and Day 7 (Fig. 2D).

Erythropoietin participates in renal protection through activating the Wnt/β-catenin pathway post kidney IRI

We observed that Wnt7b mRNA content increased rapidly within a short time and reached its peak on the 3rd day after AKI. The content thereafter gradually declined. Based on quantitative analysis, Wnt7b expression in the EPO treatment group was significantly higher than that of the vehicle

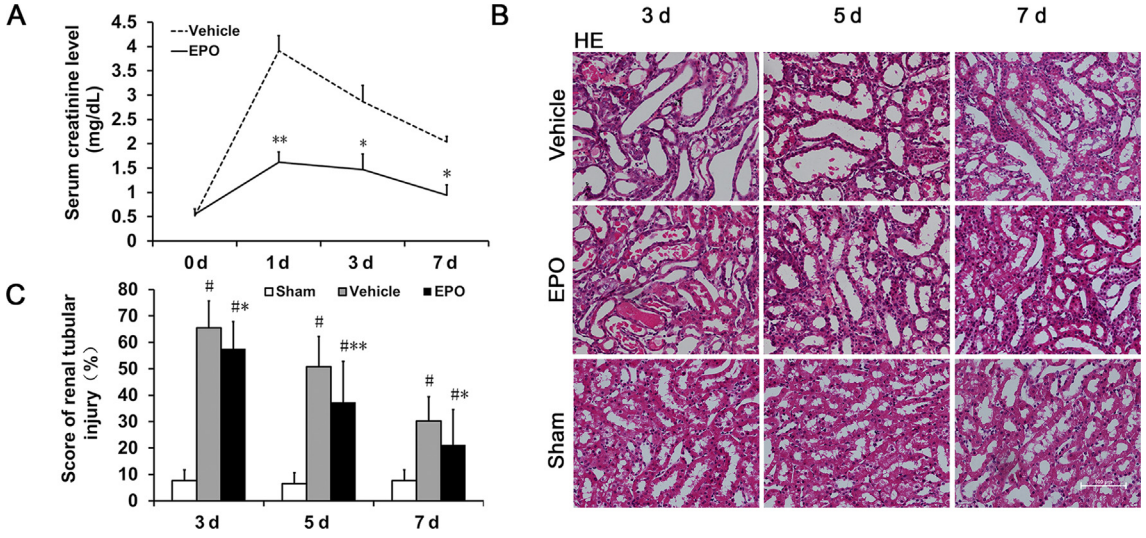

Figure 1 Erythropoietin (EPO) treatment post kidney ischemia/reperfusion injury (IRI) improves renal function and reduces tubule injury. (A) Serum creatinine levels on Day 0, Day 1, Day 3, and Day 7 after bilateral IRI, followed by intraperitoneal injection with saline or EPO 6 hours after reperfusion. (B) Representative light microscopy images of hematoxylin-eosin (HE)-stained sections of the renal outer medulla from every group (magnification, ×200). (C) The graph shows the renal tubular injury score in every group. #Indicates significance at $p < 0.01$, versus the sham group. *Indicates significance at $p < 0.05$, versus the vehicle group. **Indicates significance at $p < 0.01$, versus the vehicle group.

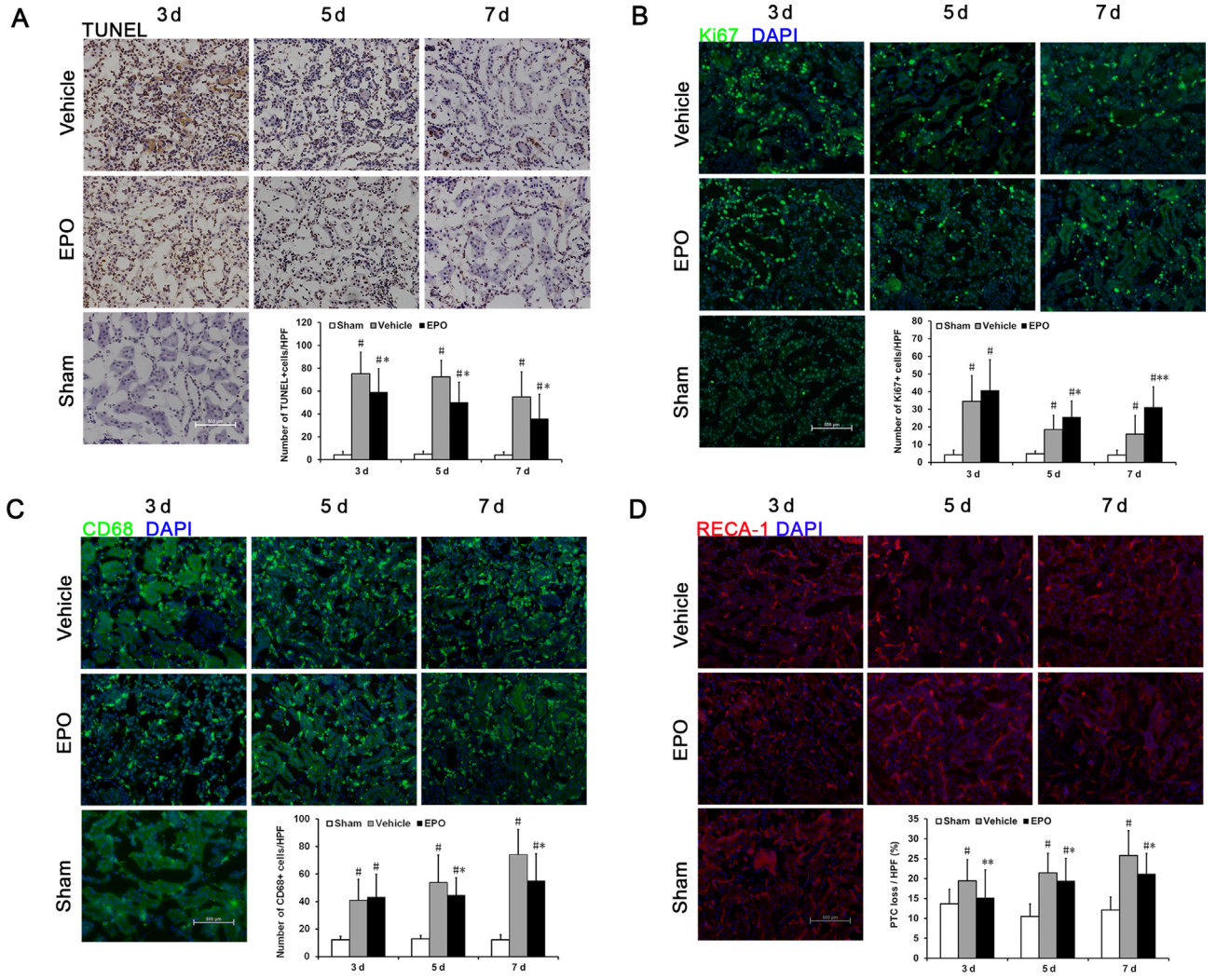

Figure 2 The administration of erythropoietin (EPO) regulates cell proliferation and apoptosis, and suppresses macrophage infiltration and peripheral capillaries loss induced by kidney ischemia/reperfusion injury (IRI). (A) Representative images of transferase dUTP nick end labeling (TUNEL) staining (brown) of the ischemia/reperfusion-injured kidney that received the vehicle or EPO on Day 3, Day 5, and Day 7 after surgery. The graph shows the number of apoptotic cells in the mice after the administration of the vehicle or EPO. (B) Representative Ki67 immunofluorescence images of ischemia/reperfusion-injured kidneys that received the vehicle or EPO at each time point. The graph shows the number of proliferative cells for mice after vehicle or EPO. (C) Representative CD68 immunofluorescence images of ischemia/reperfusion-injured kidneys that received the vehicle or EPO at each time point. The graph shows the number of macrophages in the mice after the administration of the vehicle or EPO. (D) Representative images of rat RECA-1-labeled peritubular capillaries of the outer medulla on Day 7 post ischemia/reperfusion injury of kidneys that received the vehicle or EPO at each time point. The graph shows peritubular capillary loss in the mice after the administration of the vehicle or EPO. (Magnification, $\times$200; #$p < 0.01$, vs. the sham group; *$p < 0.05$ and **$p < 0.01$, vs. the vehicle group). DAPI = 4,6-diamidino-2-phenylindole.

group on the 3rd day and 5th day after IRI (Fig. 3A). The expression of β-catenin, a key downstream signaling protein of the Wnt/β-catenin pathway, was significantly increased and peaked on the 5th day (Fig. 3B and 3C). After EPO treatment, β-catenin expression was significantly enhanced, especially on the 5th day after the kidney IRI (Fig. 3B and 3C).

Erythropoietin affects the expression of IRI-related miRNAs

Numerous studies have indicated miRNAs have an important role in regulating hypoxia, cell survival, apoptosis, inflammation, and other processes. Therefore, we investigated whether EPO administration could regulate miRNAs. In our study, we specifically examined cell apoptosis, proliferation, and fibrosis-related miRNAs such as miRNA21, miRNA214, and miRNA192. We observed that miR-21 and -214 were upregulated after the IRI. However, their expression was reduced after EPO treatment (Fig. 4A and 4B). Erythropoietin had no effect on the expression of miR-192 (Fig. 4C). We also detected hypoxia-related miR-210 and -199a. We found that increased expression of these two miRNAs after the IRI was markedly suppressed by EPO treatment (Fig. 4D and 4E).

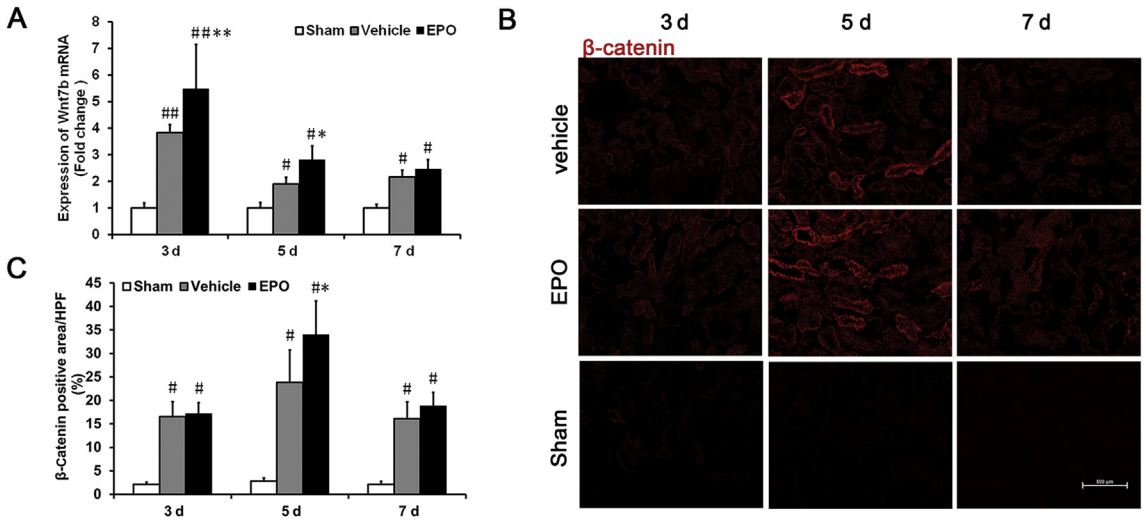

Figure 3 Erythropoietin (EPO) enhances the activation of the Wnt/β-catenin signaling pathway after ischemia/reperfusion injury (IRI) of the kidneys. (A) The graph shows the expression of a critical Wnt, Wnt7b, on Day 3, Day 5, and Day 7 post kidney IRI after vehicle or EPO administration by real-time polymerase chain reaction [PCR; normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. (B) Representative β-catenin immunofluorescence images of the ischemia/reperfusion-injured kidneys that received the vehicle or EPO at each time point. (C) The graph indicates β-catenin–positive areas for mice after the administration of vehicle or EPO (magnification ×200; #$p < 0.01$, vs. the sham group; *$p < 0.05$ and **$p < 0.01$, vs. the vehicle group).

Discussion

Acute kidney injury induced by IRI is a common clinical problem and has a mortality rate of 25–70% because of limited treatment.[20] Therefore, it is very important to seek new treatment strategies. Erythropoietin is a glycoprotein that has a critical hormonal role in regulating erythrocyte production, and it can protect a wide variety of tissues from diverse injuries in addition to its role in erythropoiesis.[4,21–23] In the present study, we demonstrated that EPO administration can significantly improve renal function, prevent tubular cell apoptosis, and promote cell proliferation after IRI. Furthermore, EPO treatment suppressed macrophage infiltration and prevented loss of the microvasculature. Furthermore, EPO clearly enhanced the expression of Wnt7b and β-catenin and regulated the expression of IRI-related miRNAs.

Erythropoietin has validated anti-inflammatory, -oxidant, and -apoptotic effects when it is used prior to ischemia.[5,24–27] However, most studies have focused on EPO preconditioning in kidney IRI. In this experiment, we aimed to observe whether, with the exception of its prophylactic

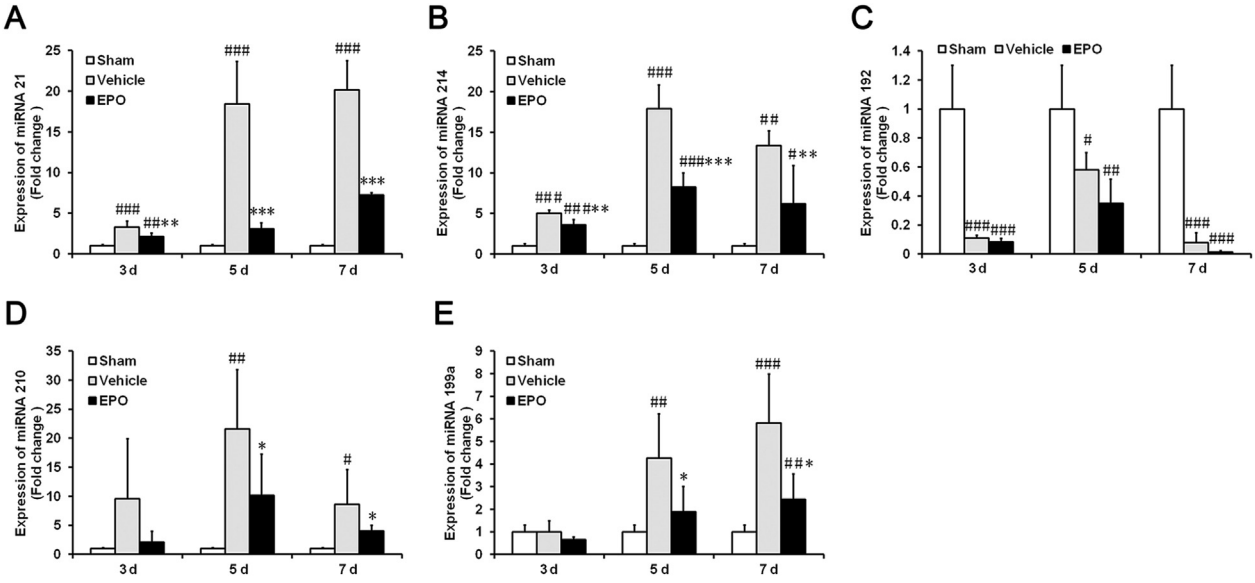

Figure 4 EPO regulates the expression of IRI-related miRNAs. (A–E) The graph shows the expression of miR-21, -214, -192, -210, and -199a on Day 3, Day 5, and Day 7, after kidney ischemia/reperfusion injury (IRI) after vehicle or erythropoietin (EPO) by real-time PCR (normalized to U6) (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$, vs. the sham group; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, vs. the vehicle group).

role, EPO treatment at an early stage could contribute to kidney repair and regeneration after injury. After treatment, the serum creatinine level decreased significantly and the renal function improved. In addition, the renal tubular damage index and cell infiltration of renal interstitial inflammation were reduced, which morphologically showed great improvement of the kidney injury. To our knowledge, EPO is prophylactic and effective is a new finding, even though it was administered during perfusion periods.

Cell necrosis and apoptosis were generated with decreased glomerular filtration rate.[28] In the present study, we observed apoptosis of renal cells in IRI model and found apoptosis was enhanced at the early stage of IRI, especially in the epithelia. The administration of EPO markedly inhibited apoptosis. This antiapoptotic effect may involve multiple mechanisms such as EPO on its receptor protecting the tubular cells,[9,29] EPO inducing the expression of heme oxygenase 1 (HO-1) against oxidative stress,[30] and EPO blocking the passing down of apoptotic signaling pathways.[31] In addition, the replacement and regeneration of renal tubular epithelial cells may be key in the recovery from kidney IRI.[32]

We found that the proliferation of tubular epithelial cells decreased with the extension of the reperfusion time. After EPO treatment, epithelial cell proliferation was enhanced during the late stage after IRI, which may result from an EPO post-treatment effect or long-term restoration.

Emerging evidence indicates that the integrity of the PTC network is a key determinant of the preservation of renal function.[33] Our study showed that the loss of PTCs increased in kidney tissue after ischemia/reperfusion, but this loss was significantly ameliorated after EPO treatment, especially after 3 days. Certain past findings have also proven that EPO has a direct biological effect on endothelial cells[34] and endothelial progenitor cells,[35] and hence contribute to the vascular repair process and vascularization.[36]

Vascular endothelial damage and dysfunction also lead to inflammatory cell infiltration.[2] In our study, we observed significant increases in macrophages. In general, macrophages have an important role in mediating injury and repair after IRI, which depends on macrophage phenotypes. During the early stages of IRI, macrophages have a proinflammatory role in mediating injury; during the late phase, they transform themselves into the anti-inflammatory phenotype to repair injury.[37,38] In this study, the increase in CD68+ macrophages could be mitigated by EPO treatment after IRI.

Our previous study and other investigations have shown that the Wnt pathway is activated and has an indispensable role during repair and regeneration in animal models of acute ischemic injury.[13,16] In this experiment, we also observed the Wnt/β-catenin signal pathway is sequentially activated after kidney IRI. Wnt7b, a Wnt pathway ligand, is a critical tissue ligand in the preparation of kidney damage. This ligand's mRNA expression increased rapidly within a short time and reached its peak on Day 3 after the acute kidney IRI. This finding indicated the process of reparation and regeneration by activation of the Wnt/β-catenin pathway.[13] To our astonishment, EPO further enhanced the repair signals of Wnt7b and β-catenin, compared to signaling in the IRI group. Erythropoietin inhibited the infiltration of macrophages, which is the major source of Wnt7b, although it did not alter the increase in expression. This may be because EPO treatment promotes the proliferation of tubular epithelial cells and endothelial cells (which are also capable of secreting Wnt7b) or because it promotes the phenotype switch of macrophages from proinflammation to proreparation. Further study needs to be investigated in the future.

Numerous hallmarks of kidney disease such as hypoxia, apoptosis, proliferation, inflammation, epithelial-mesenchymal transition, and fibrosis are regulated by miRNAs.[17–19] Research studies have confirmed that miR-21 and -214 have a role in regulating cellular proliferation, preventing apoptosis, and promoting renal fibrosis by regulating the transforming growth factor-β signaling pathway.[17,18,39–41] Our findings indicated that miR-21 and -214 were upregulated, whereas miR-192 was downregulated after the IRI. These findings were in line with the findings of a previous study.[19] In addition, EPO administration reduced the expression of miR-21 and -214, but inconspicuously influenced miR-192 expression. This finding may be because the potential roles of miR-192 in IRI and fibrosis remain controversial.[18] Furthermore, the mechanism of the regulation of miR-192 expression remains unclear. We also detected miR-210 and -199a expression, which is associated with hypoxia and angiogenesis.[42,43] In particular, we discovered that the expression of these two miRNAs increased after IRI. Erythropoietin also suppressed the overexpression of the two miRNAs. We did not find sufficient evidence of EPO directly regulating these miRNAs, although these miRNAs may represent new biomarkers of renal injury, as previously reported.[19]

In summary, our study elucidated that EPO improved renal function, reduced apoptosis, and decreased tubular injury after kidney IRI. We further confirmed that EPO promoted the regeneration of kidney tubules, and prevented PTC loss. The functional and histological benefits of EPO on the kidneys after IRI were accompanied by activation of Wnt7b/β-catenin and downregulation of miR-21, -214, -210, and -199a. Whether these findings are responsible for the renoprotective effect of EPO needs further studies. Our results strongly suggest that EPO could be a promising therapeutic strategy against the pathological development of AKI caused by IRI.

Acknowledgments

This study was supported by research grants from the National Basic Research Program of China 973 Program (No. 2012CB517602, Beijing, China), the National Natural Science Foundation of China (81370812, Beijing, China), the research fund for the Doctoral Program of Ministry of Education of China (20122307110018, Beijing, China), the Special Grade of China Postdoctoral Science Foundation (No. 201003463, Beijing, China), and the Heilongjiang Postdoctoral Science Research Foundation (No. LBHQ10028, Harbin, Heilongjiang).

References

1. Nony PA, Schnellmann RG. Mechanisms of renal cell repair and regeneration after acute renal failure. *J Pharmacol Exp Ther* 2003;**304**:905–12.

2. Lien YH, Lai LW, Silva AL. Pathogenesis of renal ischemia/reperfusion injury: lessons from knockout mice. *Life Sci* 2003;**74**:543—52.
3. Aydin Z, van Zonneveld AJ, de Fijter JW, Rabelink TJ. New horizons in prevention and treatment of ischaemic injury to kidney transplants. *Nephrol Dial Transplant* 2007;**22**:342—6.
4. Chang YT, Pan SY, Lin SL. Seeking for a way to revive erythropoietin production in chronic kidney disease. *J Formos Med Assoc* 2013;**112**:657—8.
5. Hu L, Yang C, Zhao T, Xu M, Tang Q, Yang B, et al. Erythropoietin ameliorates renal ischemia and reperfusion injury *via* inhibiting tubulointerstitial inflammation. *J Surg Res* 2012;**176**:260—6.
6. Yeh PY, Liao FL, Lin SL. Is the renoprotective effect of erythropoietin in chronic kidney disease a myth? *J Formos Med Assoc* 2013;**112**:655—6.
7. Nairz M, Sonnweber T, Schroll A, Theurl I, Weiss G. The pleiotropic effects of erythropoietin in infection and inflammation. *Microbes Infect* 2012;**14**:238—46.
8. Simon F, Scheuerle A, Calzia E, Bassi G, Oter S, Duy CN, et al. Erythropoietin during porcine aortic balloon occlusion-induced ischemia/reperfusion injury. *Crit Care Med* 2008;**36**:2143—50.
9. Sharples EJ, Patel N, Brown P, Stewart K, Mota-Philipe H, Sheaff M, et al. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J Am Soc Nephrol* 2004;**15**:2115—24.
10. Furuichi K, Kaneko S, Wada T. Chemokine/chemokine receptor-mediated inflammation regulates pathologic changes from acute kidney injury to chronic kidney disease. *Clin Exp Nephrol* 2009;**13**:9—14.
11. Namikoshi T, Satoh M, Horike H, Fujimoto S, Arakawa S, Sasaki T, et al. Implication of peritubular capillary loss and altered expression of vascular endothelial growth factor in IgA nephropathy. *Nephron Physiol* 2006;**102**:9—16.
12. Ishii Y, Sawada T, Kubota K, Fuchinoue S, Teraoka S, Shimizu A. Injury and progressive loss of peritubular capillaries in the development of chronic allograft nephropathy. *Kidney Int* 2005;**67**:321—32.
13. Lin SL, Li B, Rao S, Yeo EJ, Hudson TE, Nowlin BT, et al. Macrophage Wnt7b is critical for kidney repair and regeneration. *Proc Natl Acad Sci USA* 2010;**107**:4194—9.
14. Li B, Cohen A, Hudson TE, Motlagh D, Amrani DL, Duffield JS. Mobilized human hematopoietic stem/progenitor cells promote kidney repair after ischemia/reperfusion injury. *Circulation* 2010;**121**:2211—20.
15. Li B, Morioka T, Uchiyama M, Oite T. Bone marrow cell infusion ameliorates progressive glomerulosclerosis in an experimental rat model. *Kidney Int* 2006;**69**:323—30.
16. Kawakami T, Ren S, Duffield JS. Wnt signalling in kidney diseases: dual roles in renal injury and repair. *J Pathol* 2013;**229**:221—31.
17. Saal S, Harvey SJ. MicroRNAs and the kidney: coming of age. *Curr Opin Nephrol Hypertens* 2009;**18**:317—23.
18. Chung AC, Yu X, Lan HY. MicroRNA and nephropathy: emerging concepts. *Int J Nephrol Renovasc Dis* 2013;**6**:169—79.
19. Godwin JG, Ge X, Stephan K, Jurisch A, Tullius SG, Iacomini J. Identification of a microRNA signature of renal ischemia reperfusion injury. *Proc Natl Acad Sci USA* 2010;**107**:14339—44.
20. Rabb H, O'Meara YM, Maderna P, Coleman P, Brady HR. Leukocytes, cell adhesion molecules and ischemic acute renal failure. *Kidney Int* 1997;**51**:1463—8.
21. Liu Y, Luo B, Han F, Li X, Xiong J, Jiang M, et al. Erythropoietin-derived nonerythropoietic peptide ameliorates experimental autoimmune neuritis by inflammation suppression and tissue protection. *PLoS One* 2014;**9**:e90942.
22. Feng Q. Beyond erythropoiesis: the anti-inflammatory effects of erythropoietin. *Cardiovasc Res* 2006;**71**:615—7.
23. Rossert J, Eckardt KU. Erythropoietin receptors: their role beyond erythropoiesis. *Nephrol Dial Transplant* 2005;**20**:1025—8.
24. Ahmadiasl N, Banaei S, Alihemmati A, Baradaran B, Azimian E. The anti-inflammatory effect of erythropoietin and melatonin on renal ischemia reperfusion injury in male rats. *Adv Pharm Bull* 2014;**4**:49—54.
25. Ahmadiasl N, Banaei S, Alihemati A, Baradaran B, Azimian E. Effect of a combined treatment with erythropoietin and melatonin on renal ischemia reperfusion injury in male rats. *Clin Exp Nephrol* 2014;**18**:855—64.
26. Moeini M, Nematbakhsh M, Fazilati M, Talebi A, Pilehvarian AA, Azarkish F, et al. Protective role of recombinant human erythropoietin in kidney and lung injury following renal bilateral ischemia-reperfusion in rat model. *Int J Prev Med* 2013;**4**:648—55.
27. Caetano AM, Vianna Filho PT, Castiglia YM, Golim MA, de Souza AV, de Carvalho LR, et al. Erythropoietin attenuates apoptosis after ischemia-reperfusion-induced renal injury in transiently hyperglycemic Wister rats. *Transplant Proc* 2011;**43**:3618—21.
28. Gobe G, Willgoss D, Hogg N, Schoch E, Endre Z. Cell survival or death in renal tubular epithelium after ischemia-reperfusion injury. *Kidney Int* 1999;**56**:1299—304.
29. Spandou E, Tsouchnikas I, Karkavelas G, Dounousi E, Simeonidou C, Guiba-Tziampiri O, et al. Erythropoietin attenuates renal injury in experimental acute renal failure ischaemic/reperfusion model. *Nephrol Dial Transplant* 2006;**21**:330—6.
30. Katavetin P, Inagi R, Miyata T, Shao J, Sassa R, Adler S, et al. Erythropoietin induces heme oxygenase-1 expression and attenuates oxidative stress. *Biochem Biophys Res Commun* 2007;**359**:928—34.
31. Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, et al. Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. *Circ Res* 2002;**90**:745—8.
32. Esson ML, Schrier RW. Diagnosis and treatment of acute tubular necrosis. *Ann Intern Med* 2002;**137**:744—52.
33. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest* 2011;**121**:4210—21.
34. Goldman SA, Nedergaard M. Erythropoietin strikes a new cord. *Nat Med* 2002;**8**:785—7.
35. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. *Early Hum Dev* 1998;**52**:235—49.
36. Metivier F, Marchais SJ, Guerin AP, Pannier B, London GM. Pathophysiology of anaemia: focus on the heart and blood vessels. *Nephrol Dial Transplant* 2000;**15**(Suppl. 3):14—8.
37. Kinsey GR. Macrophage dynamics in AKI to CKD progression. *J Am Soc Nephrol* 2014;**25**:209—11.
38. Duffield JS. Macrophages in kidney repair and regeneration. *J Am Soc Nephrol* 2011;**22**:199—201.
39. Denby L, Ramdas V, McBride MW, Wang J, Robinson H, McClure J, et al. miR-21 and miR-214 are consistently modulated during renal injury in rodent models. *Am J Pathol* 2011;**179**:661—72.
40. Chau BN, Xin C, Hartner J, Ren S, Castano AP, Linn G, et al. MicroRNA-21 promotes fibrosis of the kidney by silencing metabolic pathways. *Sci Transl Med* 2012;**4**:121ra18.
41. Denby L, Ramdas V, Lu R, Conway BR, Grant JS, Dickinson B, et al. MicroRNA-214 antagonism protects against renal fibrosis. *J Am Soc Nephrol* 2014;**25**:65—80.
42. McCormick RI, Blick C, Ragoussis J, Schoedel J, Mole DR, Young AC, et al. miR-210 is a target of hypoxia-inducible factors 1 and 2 in renal cancer, regulates ISCU and correlates with good prognosis. *Br J Cancer* 2013;**108**:1133—42.
43. Rane S, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ Res* 2009;**104**:879—86.