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Remote limb ischemic preconditioning (rIPC) activates antioxidant and antiapoptotic genes and inhibits proinflammatory cytokine genes in renal ischemia/reperfusion injury

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Abstract. The mechanisms underlying the renoprotective effect for remote limb ischemic preconditioning (rIPC) against renal ischemia/reperfusion injury need further elucidation. In our work, one hundred and twenty male Sprague Dawley rats were randomized into 3 groups; sham, I/R group (left renal 45 min ischemia) and rIPC (as I/R group with 3 cycles of left femoral ischemic PC just before renal ischemia). Rats were sacrificed at 2 h, 24 h, 48 h and 7 days. Serum creatinine and urea were measured at the baseline and endpoints. Also, histopathological examination and assessment of the expression of inflammatory cytokines e.g. *TNF-α, IL-1β* and *ICAM-1* and antioxidant genes: *Nrf2, HO-1 and NQO-1* and anti-apoptotic gene *Bcl-2* in left kidney were done by the end of experiment. The results of this study demonstrated that, rIPC caused significant improvement in serum creatinine and BUN levels and in the expression of antioxidant genes and *Bcl-2* antiapoptotic gene with significant attenuation of pro-inflammatory cytokines and histopathological damage score at all-time points compared to I/R group ($p \leq 0.05$). In conclusion, inhibition of inflammatory cytokine (*TNF-α, IL-1β* and *ICAM-1*) formation and activation of antioxidant genes: *Nrf2, HO-1* and *NQO-1* and anti-apoptotic gene *Bcl-2* could be possible underlying mechanisms for the renoprotective effect of rIPC.

Key words: Remote ischemic preconditioning — Ischemia/reperfusion — Nrf2 — HO-1— NQO-1— Cytokines

Introduction

Renal ischemia/reperfusion (I/R) is a commonly encountered clinical scenario in urological practice especially during nephron-sparing surgery and renal transplantation. Unfortunately, the I/R process is associated with varying degrees of kidney damage not only because of the ischemic injury, but also as reperfusion is associated with the release of reactive oxygen species and pro-inflammatory mediators (Rodriguez et al. 2013). Extensive efforts have been done to alleviate the harmful effects of renal I/R injury by pharmacological and non-pharmacological methods such as ischemic preconditioning in which brief episodes of ischemia followed by reperfusion done before the prolonged definite ischemia (Gassanov et al. 2014). Recent studies by our group showed protective effect for local ischemic preconditioning against renal I/R (Shokeir et al. 2012, 2014). Furthermore, it has been shown that this protective effect was obtained even when the brief episodes of ischemia were applied to a remote organ, commonly a limb before the definite organ ischemia; a term called remote ischemic preconditioning (rIPC) (Candilio et al. 2013).

Adequate description of the underlying mechanisms by which renoprotection is obtained after rIPC is mandatory to improve the outcome and optimize the effect. Previous reports concluded that an intra-renal mechanism acting within cortical cells might underpin the renoprotective func-

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tion of rIPC (Gardner et al. 2014), in addition, this protection might be a consequence of reductions in lipid peroxidation, intensification of anti-oxidant systems and downregulation of COX-2 expression (Sedaghat et al. 2013). Furthermore, this renoprotective effect might be attributed to endorphin release from the ischemic limb (Wever et al. 2013). Although several studies have demonstrated the renoprotective effect of rIPC (Huang et al. 2013; Igarashi et al. 2013), the exact mechanisms have to be elucidated. Moreover, a recent study by our group demonstrated the role of antioxidant genes *Nrf2, HO-1* and *NQO-1* as endogenous adaptive mechanisms that explain the renoprotective actions of local IPC and postconditioning against renal I/R injury (Shokeir et al. 2014). We hypothesized that remote IPC might share the same protective mechanisms of local IPC. So, in this study, we examined the effect of rIPC on renal I/R injury as well as the possible role for antioxidant genes (*Nrf2, HO-1* and *NQO1*) antiapoptotic (*Bcl-2*) gene and pro-inflammatory cytokines (TNF-α, IL-1β and ICAM-1) as potential mechanisms by which rIPC could protect the kidney from I/R injury.

Materials and Methods

Experimental animals

One hundred and twenty male Sprague Dawley rats weighing 200–250 g (aged 4–6 months) were enrolled in this study. Animals were bred in the animal research facility in the Urology & Nephrology Center at Mansoura, Egypt. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, no. 85-23, revised 1996) and all protocols were approved by our ethical committee of Mansoura, Faculty of Medicine.

Experimental design

Rats were divided into 3 groups (40 rats each); a) sham group; left kidney renal pedicle was surgically explored without ischemia with right nephrectomy, b) I/R group, clamping of left renal pedicle for 45 min with right nephrectomy and c) rIPC group: as I/R group with remote limb ischemic preconditioning on left hind limb. Each group was further subdivided into 4 subgroups (10 each) according to the time of their sacrifice; 2 h, 24 h, 48 h and 7 days.

Experimental procedures

In all rats, anesthesia was induced by *intraperitoneal* (*i.p.*) injection of a mixture of ketamine 75 mg/kg and diazepam 5 mg/kg. In sham operated group, a midline laparotomy was performed, the left kidney and its pedicle was dissected off the surrounding perirenal fat along the renal surface, and then exposed for 45 min without vascular clamping. Then, right renal vascular pedicle was exposed and ligated using 3-0 silk sutures and the kidney was removed. The abdomen was irrigated with isotonic saline and the abdominal incision was closed using polyglactin 2/0 sutures. In control group, the same procedures were done in addition to clamping of the left renal pedicle for 45 min, then right nephrectomy 5 min before the release of the left renal vascular pedicle (Shokeir et al. 2012). For rIPC group, the same procedures were done in addition to three interspersed cycles of ischemia (for 5 min) and reperfusion (for 5 min) with an elastic band tourniquet applied at the base of left hind limb of the animal just before the definite renal ischemia.

Collection of blood samples and harvesting kidney tissues

Blood samples (1 ml) were obtained from the ophthalmic venous plexus of each rat using a fine walled Pasteur pipette before surgical procedure (basal) and before the time of sacrifice (at 2 h, 24 h, 48 h and 7 days) (test). Under halothane anesthesia, the pipette was introduced gently at the inner side of the eyeball and pushed firmly along the side of the orbit to the ophthalmic venous plexus. The blood sample was centrifuged and the serum was isolated and stored at –20°C for measurement of serum creatinine and blood urea nitrogen (BUN).

By the end of experiment for each rat, the rat was anesthetized by large dose of Na^+ thiopental (120 mg/kg), then abdomen was rapidly opened and left kidney was harvested and bisected into 2 halves, one half was placed and fixed in 10% formalin solution for histopathological examination and the second half was stored at –80°C for real time PCR assessment of pro-inflammatory cytokines, anti-apoptotic and antioxidant genes.

Measurements of renal function

Serum creatinine and BUN levels were measured by using auto-analyzer (CX 7; Beckman, Foster City, CA, USA).

Real time PCR for inflammatory cytokines (TNF-α, IL-1β and ICAM-1), antiapoptotic (Bcl2) and antioxidant (Nrf-2, HO-1 and NQO-1) genes
RNA extraction and cDNA synthesis

According to the manufacturer's instructions, total RNA from kidney tissue specimens was isolated by disruption of 50–100 mg tissues in 1 ml of Trizol (Invitrogen Corporation, Grand Island, NY, USA). RNA was quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. Only

samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for real-time RT-PCR. Reverse transcription was done using 1 μg total RNA and a cDNA kit (high-capacity cDNA archive kit). The primer sequences for tested genes were: TNFα (295 bp) forward 5'-TACTGAACTTCGGGGTGATTGGTCC-3' reverse 5'-CAGCCTTGTCCCTTGAAGAGAACC-3', ICAM-1 (409 bp) 5'-TGTTTCCTGCCTCTGAAGC-3', IL-1β (131 bp), forward: 5'-TGTGATGTTCCCATTAGAC-3', reverse: 5'-AATACCACTTGTTGGCTTA-3', Bcl2 forward 5'-ATCGCTCTGTGGATGACTGAGTAC, reverse 5'-AGAGACAGCCAGGAGAAATCAAAC, Nuclear erythroid-related factor 2 (Nrf2) (109 bp), forward: 5'-GCTATTTTCCATTCCCGAGTTAC-3', reverse: 5'-ATTGCTGTCCATCTCTGTCAG-3'. NAD (P) H: quinine oxidoreductase-1 (NQO1) (197 bp), forward: 5'-CATCATTTGGGCAAGTCC-3', reverse: 5'-ACAGCCGTGGCAGAACTA-3'. Heme oxidase-1 (HO-1) (102 bp), forward: 5'-CTTTCAGAAGGGTCAGGTGTC-3', reverse: 5'-TGCTTGTTTCGCTCTATCTCC-3', GAPDH (140 bp) forward: 5'-TATCGGACGCCTGGTTAC-3', reverse: 5'-CTGTGCCGTTGAACTTGC-3'.

Real time PCR reaction

The reaction was performed in a total volume of 50 μl containing 25 μl from 1 × TaqMan® Universal PCR with 2.5 μl from 20 × TaqMan® Gene Expression Assay Mix and 22.5 μl of cDNA diluted in RNase-free water. The cycling parameters were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation 95°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute. Data analysis was carried out using ABI prism 7000 by equation $2^{-\Delta\Delta}$ ct (Livak and Schmittgen 2001).

Histopathological examination

After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. For histopathologic assessment of ischemic tubular injury, we used a modified form of well-established grading scales (scores of 0 to 4) (Solez et al. 1974). The numerical score used as follows: 0 indicates no damage; 1, unicellular patchy isolated necrosis; 2, tubular necrosis <25%; 3, tubular necrosis between 25% and 50%; 4, tubular necrosis >50%.

Immunohistochemical examination of Nrf2 and TNF-α

For immunohistochemistry, 3 μm-thick sections were prepared on charged slides from paraffin blocks and deparaffinized. All sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol and microwave-heated in 10 mM citrate buffer, pH 6.0, for 10–20 min. Subsequently, an indirect immunoperoxidase technique was applied, using antibodies such as anti-Nrf2 polyclonal antibody (catalog # sc-13032 X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-TNFα polyclonal antibody (catalog # sc-1350; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Indirect immunoperoxidase was performed using ImmunoPure UltraSensitive ABC Peroxidase (catalogue number: 32052; Thermo Scientific, Waltham, MA, USA) with 3,3'-diaminobenzidine (DAB) as chromogen. Proper positive and negative controls were performed. Placenta was used as positive control for Nrf2. As a negative control, sections were stained without the addition of a primary antibody.

Statistical analysis

All statistical analysis was performed using IBM SPSS statistical software (version 20). The results were expressed as mean ± SD. The comparison between baseline and endpoint measures were tested using paired sample T test. Also, one away ANOVA and Tukey's post hoc tests were used to find statistical significance among various groups in all studied parameters at the end points ($p \leq 0.05$ is considered significant).

Results

Effects of rIPC on kidney functions (serum creatinine (mg/dl) and BUN (mg/dl))

The mean values of serum creatinine and BUN were comparable in different studied groups at basal levels and no statistical significant differences among the 3 groups at basal level. The mean values of endpoints of serum creatinine and BUN increased significantly in all groups compared to their basal values ($p \leq 0.01$). The endpoint values of serum creatinine and BUN were significantly high in I/R group compared to sham group at different times except at 2 h ($p < 0.001$). Moreover, the endpoint values of serum creatinine and BUN in rIPC group were significantly lower in rIPC group compared to I/R group at different times except at 2 h ($p \leq 0.035$) (Table 1).

Effects of rIPC on the expression of pro-inflammatory cytokines (TNF-α, ICAM-1 and IL-1β) and anti-apoptotic (Bcl-2) genes

The expression of pro-inflammatory cytokines (TNF-α, ICAM-1 and IL-1β) at the level of mRNA showed significant increase in I/R group compared to sham group ($p < 0.001$) and the maximal increase in their expression was at 48 h.

Table 1. Baseline and endpoint serum creatinine (mg/dl) and BUN (mg/dl) at 2 h, 24 h, 48 h and 7 days for sham, I/R and rIPC group

	2 h		24 h		48 h		7 days	
	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
Serum creatinine (mg/dl)								
Sham group	0.41 ± 0.11	0.81 ± 0.15*	0.42 ± 0.13	0.85 ± 0.19*	0.5 ± 0.12	0.68 ± 0.13*	0.41 ± 0.07	0.58 ± 0.07*
I/R group	0.52 ± 0.12	0.81 ± 0.09*	0.45 ± 0.08	3.50 ± 0.8*,a	0.61 ± 0.05	1.70 ± 0.67*,a	0.5 ± 0.1	1.0 ± 0.30*,a
rIPC group	0.52 ± 0.12	0.8 ± 0.09*	0.45 ± 0.08	1.69 ± 0.4*,a,b	0.61 ± 0.05	1.0 ± 0.17*,a,b	0.5 ± 0.1	0.74 ± 0.20*,a,,b
Serum blood urea nitrogen (BUN) (mg/dl)								
Sham group	17.5 ± 4.60	27.4 ± 3.90*	23.3 ± 5.50	43 ± 8.80*	16.9 ± 3.50	22.6 ± 3.20*	18.4 ± 3.60	23.6 ± 3.20*
I/R group	21.4 ± 3.70	27.0 ± 3.0*	19.5 ± 3.0	62.0 ± 12.50*,a	19.0 ± 2.70	49.7 ± 6.10*,a	17.70 ± 4.0	41.0 ± 5.30*,a
rIPC group	21.4 ± 3.70	27.0 ± 3.10*	24.9 ± 4.60	51.50 ± 11.0*,a,b	19.0 ± 2.70	39.7 ± 6.10*,a,b	21.0 ± 2.0	25.10 ± 2.80*,b

All data were expressed as mean ± SD. * ignificant *vs.* baseline value of the same group (paired T-test), [a] significant *vs.* sham group of the same time period, [b] significant *vs.* I/R group of the same time period (one way ANOVA and Tukey's post hoc test). I/R, ischemia/reperfusion; rIPC, remote ischemic preconditioning.

This increase was significantly attenuated in rIPC group compared to I/R group at different times of study ($p < 0.001$). Their expression showed no statistical significant difference between sham and rIPC groups throughout the study endpoints ($p > 0.05$) (Table 2).

Also, the expression of anti-apoptotic gene *Bcl-2* showed significant decrease in I/R group compared to sham group ($p < 0.001$) and this reduction was significantly increased in rIPC group ($p < 0.001$) (Table 2).

Effects of rIPC on the expression of antioxidant (Nrf2, HO-1 and NQO-1) genes

The expression of antioxidant genes (*Nrf2, HO-1 and NQO1*) showed significant increase in I/R group compared to sham group through the study endpoints ($p < 0.001$) and their expression in rIPC group showed more significant increase in rIPC group compared to I/R group ($p \leq 0.01$) (Table 3). Immunohistochemical examination revealed minimal nuclear expression for *Nrf2* in sham group with average score 2% (Fig. 1a), increased nuclear expression for Nrf2 in I/R with average score 15% (Fig. 1b) and marked expression for Nrf2 in rIPC with average expression 27% (Fig. 1c).

Effects of rIPC on histopathological damage

Histopathological damage score showed significant increase in the damage score in I/R group compared to sham group ($p < 0.001$) and rIPC group showed significant de-

Table 2. The expression of proinflammatory cytokines (*TNF-α, ICAM-1, and IL-1β*) and anti-apoptotic (*Bcl-2*) genes at the level of mRNA (by real time PCR) 2 h, 24 h, 48 h and 7 days in the sham, I/R and rIPC group

		2 h	24 h	48 h	7 days
TNF-α	Sham group	1.12 ± 0.07	1.19 ± 0.12	1.42 ± 0.25	1.3 ± 0.25
	I/R group	9.2 ± 0.48[a]	10 ± 0.46[a]	11.8 ± 0.33[a]	7.48 ± 0.55[a]
	rIPC group	1.30 ± 0.04[b]	1.36 ± 0.05[b]	1.42 ± 0.07[b]	1.18 ± 0.05[b]
ICAM-1	Sham group	0.84 ± 0.09	0.84 ± 0.1	0.85 ± 0.08	0.77 ± 0.11
	I/R group	2.7± 0.38[a]	3.59 ± 0.41[a]	4.8 ± 0.56[a]	2.2 ± 0.29[a]
	rIPC group	0.88 ± 0.11[b]	0.87 ± 0.07[b]	0.84 ±0.05[b]	0.73 ± 0.04[b]
IL-1β	Sham group	0.82 ± 0.03	0.89 ± 0.11	0.82 ± 0.08	0.99 ± 0.12
	I/R group	2.0 ± 0.1[a]	2.6 ± 0.14[a]	3.1 ± 0.08[a]	1.50 ± 0.31[a]
	rIPC group	0.93 ± 0.06[b]	0.92 ± 0.09[b]	0.88 ± 0.06[b]	0.97 ± 0.06[b]
Bcl-2	Sham group	3.48 ± 0.1	3.7 ± 0.34	3.6 ± 0.25	3.84 ± 0.36
	I/R group	2.0 ± 0.13[a]	1.12 ± 0.27[a]	0.55 ± 0.09[a]	2.3 ± 0.32[a]
	rIPC group	3.48 ± 0.23[b]	4.13 ± 0.28[b]	4.12 ± 0.42[b]	4.5 ± 0.23[a,b]

All data were expressed as mean ± SD. One way ANOVA test with post hoc Tukey's test, [a] significant *vs.* sham group of the same time period, [b] significant *vs.* I/R group of the same time period. TNF-α, tumor necrosis factor alpha; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin 1 beta; rIPC, remote ischemic preconditioning. GAPDH was used as internal control reference gene.

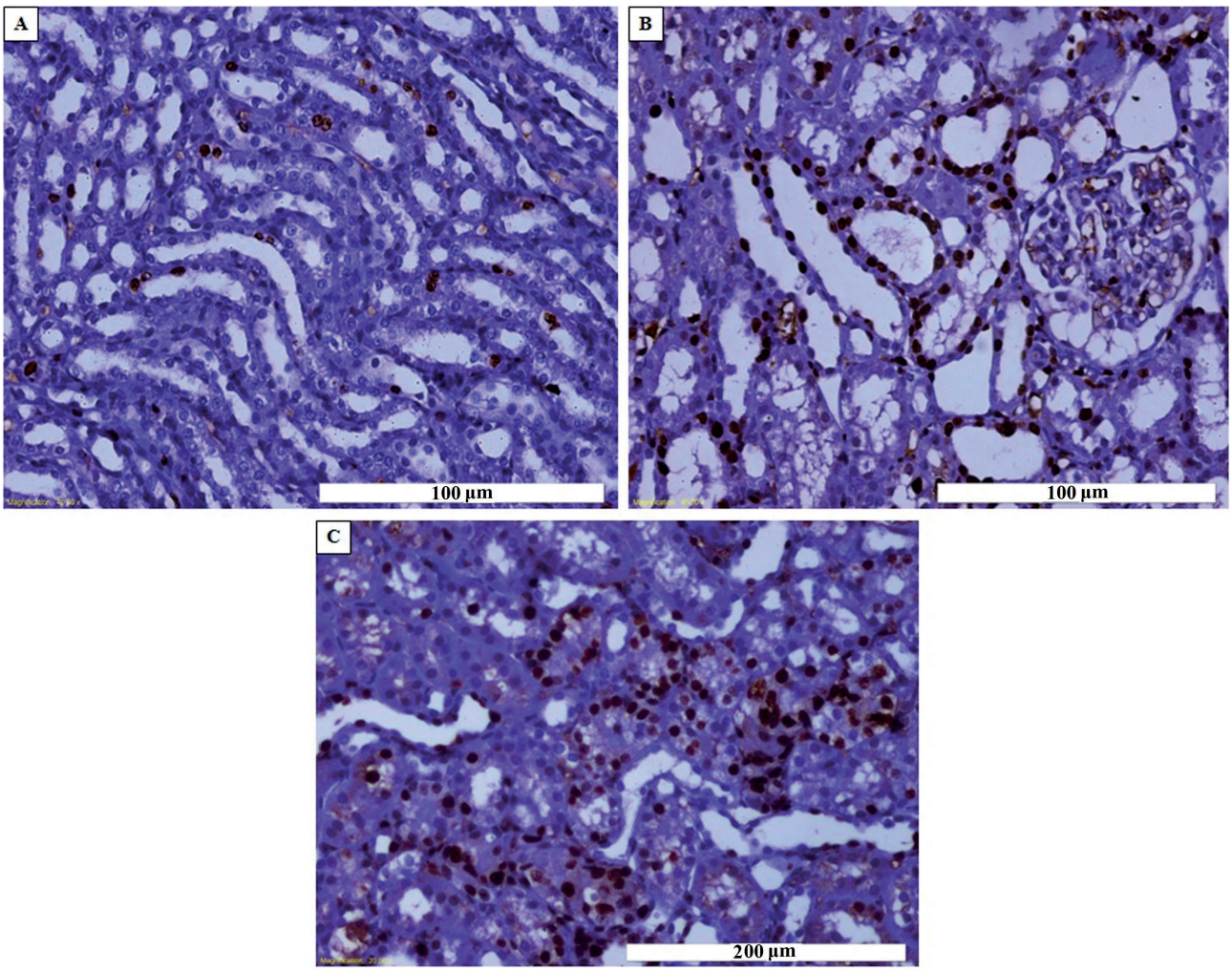

Figure 1. Kidney sections stained immunohistochemically with anti-Nrf2 antibody from sham group at 48 h with Nrf2 score 2% (**A**), I/R group at 48 h with Nrf2 score 15% (**B**), and rIPC group at 48 h with Nrf2 score 27% (**C**) protein (immunoperoxidase DAB ×200).

Table 3. The expression of antioxidants (*Nrf2, HO-1, NQO1*) genes at the level of mRNA (by real time PCR) 2 h, 24 h, 48 h and 7 days in the sham, I/R and rIPC group

		2 h	24 h	48 h	7 days
Nrf2	Sham group	0.75 ± 0.23	0.89 ± 0.24	0.84± 0.19	0.76 ± 0.18
	I/R group	2.35 ± 0.40[a]	2.78 ± 0.23[a]	2.41 ± 0.43[a]	2.52 ± 0.38[a]
	rIPC group	3.33 ± 0.67[a,b]	4.91 ± 0.81[a,b]	6.08 ± 0.95[a,b]	8.02 ± 0.97[a,b]
HO-1	Sham group	1.63 ± 0.31	1.30 ± 0.51	0.96 ± 0.11	0.97 ± 0.10
	I/R group	3.19 ± 0.63[a]	3.47 ± 0.85[a]	3.98 ± 0.30[a]	4.21 ± 0.45[a]
	rIPC group	4.21 ± 0.45[a,b]	5.74 ± 0.64[a,b]	6.63 ± 0.53[a,b]	6.16 ± 0.46[a,b]
NQO1	Sham group	1.68 ± 0.29	1.52 ± 0.39	1.12 ± 0.30	1.38 ± 0.44
	I/R group	3.52 ± 0.56[a]	3.99 ± 0.59[a]	4.21 ± 0.52[a]	4.34 ± 0.75[a]
	rIPC group	4.25 ± 0.33[a,b]	6.46 ± 0.81[a,b]	7.24 ± 0.66[a,b]	7.44 ± 0.55[a,b]

All data were expressed as mean ± SD. One way ANOVA test with post hoc Tukey's test, [a] significant *vs.* sham group of the same time period, [b] significant *vs.* I/R group of the same time period. Nrf-2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxidase-1; NQO1, NAD (P) H quinone oxidoreductase; rIPC, remote ischemic preconditioning. *GAPDH* was used as internal control reference gene.

crease in damage score compared to I/R group (Fig. 2A). Kidneys from sham rats showed normal preserved kidney structure (Fig. 2B). On the other hand, kidneys obtained from I/R group showed severe acute tubular necrosis in the cortex and the outer stripe of the outer medulla (OSOM), which included widespread degeneration of tubular architecture, detachment of epithelial cells from the basement membrane, tubular cell necrosis, intratubular cast formation and luminal congestion with extensive loss of brush border (Fig. 2C). Moreover, kidney sections obtained from the rIPC group showed a marked reduction in the histological features of renal injury consisting of mild individual tubular necrosis and minimal tubular dilatation (Fig. 2D).

Discussion

Remote ischemic preconditioning is an emerging simple and safe tool that might protect against the harmful effects of I/R injury with some benefits have been translated into clinical studies (Huang et al. 2013; Park et al. 2013; Li et al. 2013; Gardner et al. 2014; McCafferty et al. 2014). Nevertheless, the adequate underlying pathophysiological mechanisms are not fully understood. The findings of the present study confirmed the renoprotective benefit of rIPC as evidenced by significant lowering of serum creatinine and BUN values and significant improvement of histopathological damage score in rIPC group compared to I/R group. In addition, there was a significant decrease in the expression of pro-inflammatory

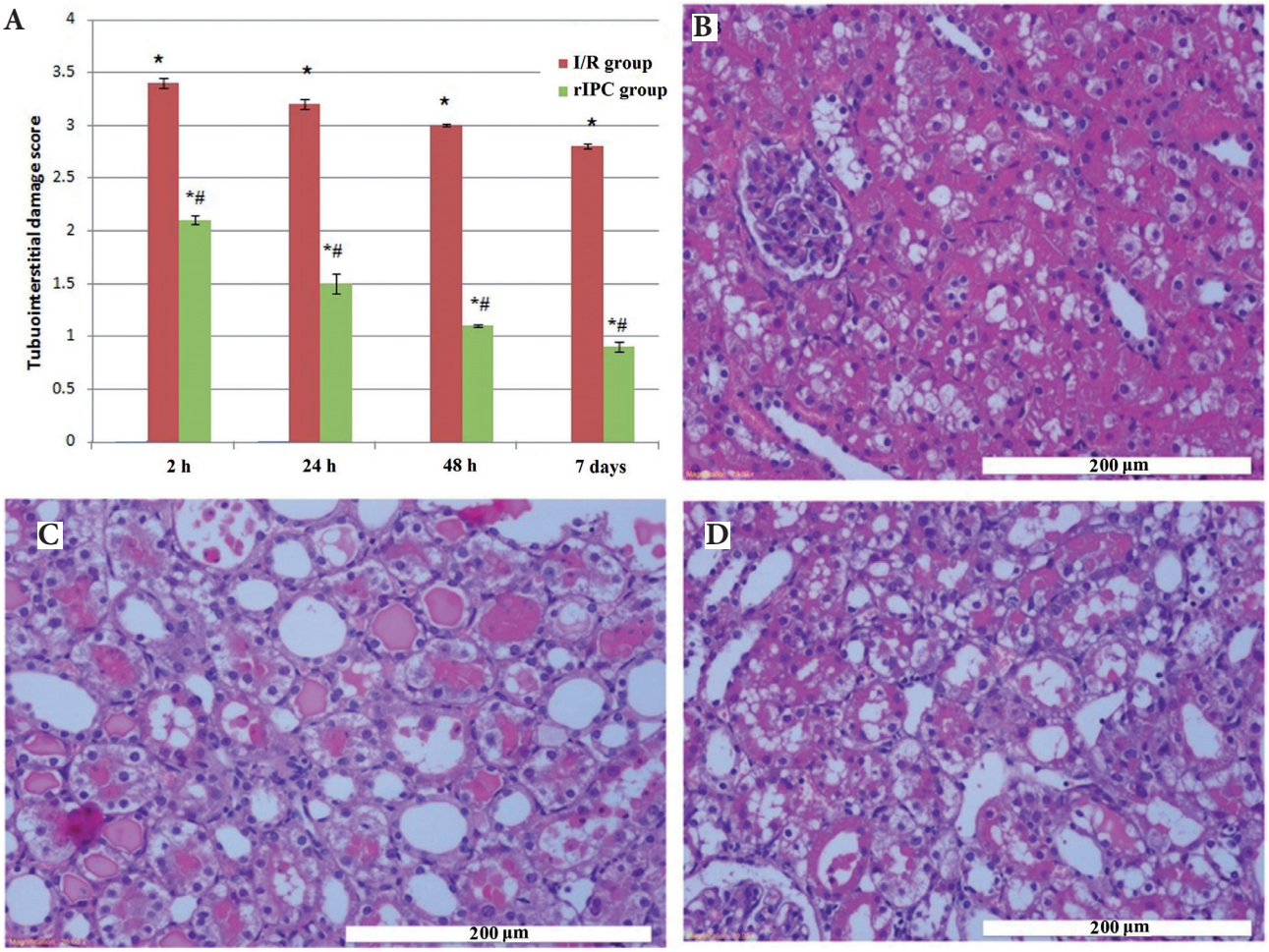

Figure 2. A. Histopathological damage score of kidney tissues in different groups (* significant *versus* sham group of the same time period, # significant *versus* I/R group of the same time period). **B**. Photomicrograph of rat renal outer strip of outer medulla (OSOM) showing normal kidney architecture (sham group at 7 days H&E ×250). **C.** Tubular dilatation, epithelial cells attenuation, necrosis, and apoptosis and tubular hyaline casts (control group 7 days H&E ×250). **D**. Few tubular dilatations, hydropic degeneration of tubular cells (rIPC 7 days H&E ×250).

cytokines (*IL-1β, TNF-α* and *ICAM-1*) genes and enhanced expression of antioxidant (*Nrf2, HO-1 and NQO-1*) genes and the anti-apoptotic (*Blc-2*) gene products in the in rIPC group compared with I/R group.

Prolonged tissue ischemia leads to irreversible death; therefore, reperfusion is mandatory to salvage tissue from inevitable necrosis. However, tissues reperfusion is associated with augmented respiratory burst and inflammation that might contribute for further tissue damage. Therefore, over expression of pro-inflammatory cytokines is expected to be one of the major determinant mechanisms for I/R injury (Friedewald and Rabb 2004; Awad and El-Sharif 2011). It has been reported that over expression of the pro-inflammatory cytokine TNF superfamily was associated with activation of the apoptotic pathways and eventually cell death (Gaur and Aggarwal 2003). In this study, *TNF-α* gene showed significant increase in its expression (at the level of mRNA) in kidney tissues obtained from I/R group compared to those obtained from sham group. This finding was in agreement with the results of early study done by our group (Shokeir et al. 2014). Moreover, rIPC caused significant attenuation of the expression of these genes. This highlights the possible role of rIPC in controlling I/R *via* anti-inflammatory effect by attenuating the expression of TNF-α. A similar effect for TNF-α has been reported by Dong et al. (2013) who concluded that vitexin might alleviate the cardiac I/R injury by decreasing the expression of TNF-α induced by myocardial I/R injury.

The adherence of neutrophils and macrophage to the endothelial surface was shown to be mediated with up-regulation of intercellular adhesion molecule-1 (ICAM-1) (Okusa 2002). Li et al. (2014) investigated the potential role of tea polyphenols in alleviating I/R injury. The researchers found tea polyphenol pretreatment attenuated the increased level of serum TNF-α, IL-β, IL-6 and ICAM-1. The same results were obtained in the current study. We have found decreased expression of the pro-inflammatory cytokines (TNF-α, IL-1β and ICAM-1) in the sham and rIPC groups *vs*. significant expression in the control group suggesting a potential role of rIPC in alleviating I/R injury though suppression of the inflammatory pathways.

Remote ischemic preconditioning has been shown to reduce the effect of I/R injury by intensification of the antioxidants system and reduction of lipid peroxidation (Sedaghat et al. 2013). Sedaghat et al. (2013) reported increases in superoxide dismutase and catalase activity, increased reduced glutathione levels and decreased malondialdehyde levels and cycologygenase-2 expression. The transcription factor Nrf2 regulates the expression of several antioxidant and cytoprotective genes and have been shown protective effects after renal I/R injury (Liu et al. 2009). In addition, the underlying mechanisms by which several antioxidant factors contributed to alleviation of I/R injury were attributed directly to the activation of *Nrf2* gene (Wu et al. 2011; Zuniga-Toala et al. 2013). We reported in the present study enhanced expression of *Nrf2* gene products at the levels of mRNA and protein levels by rIPC. Similar findings reported by our group but by local ischemic preconditioning (Shokeir et al. 2014), findings suggested that remote IPC could share the same protective mechanisms of local IPC. The enzyme NQO-1 is an important protein that guard against reactive oxygen species. It has been shown that genetic deletion of NQO-1 accelerates I/R-induced renal damage in the kidney; in addition, pharmacological activation of NQO-1 attenuates I/R injury-induced acute renal failure (Gang et al. 2014). Similarly, HO-1 enzyme was the underlying mechanism by which hyperbaric oxygen alleviated I/R injury (He et al. 2011). In addition, genetically modified macrophages expressing HO-1 have been shown to improve renal function after I/R injury (Ferenbach et al. 2010). This study examined the role of two important antioxidant enzymes, HO-1 and NOQ-1 as well as the transcription factor Nrf2 in alleviating I/R injury. We found that the expression of these antioxidant genes was enhanced in rIPC group compared to I/R group. These findings suggested involvement of the enzymes and Nrf2 transcription factor in the renoprotective mechanism of rIPC against renal I/R injury. The same results were previously reported by Shokeir et al. (2014) who have shown that *Nrf-2, HO-1* and *NQO-1* genes played an important role in alleviating ischemic preconditioning when compared to ischemic post-conditioning.

Bcl-2 is an anti-apoptotic molecule whose levels are normally decreased by T-cell activation and has been shown to be over-expressed by the presence of oxidative stress (Hildeman et al. 2003). It has been shown that reactive oxygen species induced apoptosis was induced by regulating the phosphorylation and ubiquitination of Bcl-2 proteins, resulting in increased proapoptotic protein levels and decreased anti-apoptotic protein expression (Li et al. 2004). In this study, we demonstrated increased expression of *Bcl-2* gene in the rIPC group and sham group with significant decrease in expression in control group. These findings suggest the possible underlying mechanisms of rIPC in alleviating I/R injury by activating *Bcl-2* gene transcription.

Albeit rIPC is a feasible and clinically applicable procedure (Gardner et al. 2014; Gassanov et al. 2014; Haji Mohd Yasin et al. 2014), the effects on alleviating I/R injury should be compared with standard ischemic preconditioning. Zhang et al. (2013) reported that both ischemic preconditioning and rIPC produced powerful inhibition of pro-inflammatory markers when compared to I/R injury group. However, the effect of ischemic preconditioning was more potent in alleviating myocardial I/R injury. This point need further to be assessed.

The present study lacks the description of many other possible mechanisms of which rIPC might protect against

I/R injury. However, to the best of our knowledge this study is the first study which described involvement of *Nrf2* gene in the renoprotective mechanisms rIPC against renal I/R injury. In fact, the exact mechanisms for remote inter-organ cross protection are not well-understood. It has been suggested that extracellular vesicles e.g. exosomes and microparticles, by their mRNA and microRNA contents, are ideal conveyers for inter-organ communications (Giricz et al. 2014). In addition, Shan et al. (2013) reported a decrease in cerebral infarction mediated by platelets-derived microparticles after hind limb ischemia. Opioid receptors have been suggested to play a role in rIPC. Wever et al. (2013) reported that pre-treatment with the opioid receptor antagonist naloxone completely blocked the protective effect of rIPC suggesting that the release of endorphin induced by brief hind limb ischemia might have been underlying mechanism. Furthermore, Surendra et al. (2013) demonstrated that rIPC cardioprotection required the activation of delta-opioid and kappa-opioid receptors and functional integration with adenosine A1 receptors.

Decreased platelet activity and aggregation is a hypothesized mechanism that might contribute to the beneficial effects of rIPC. Stazi et al. (2013) showed that rIPC before radiofrequency catheter ablation for atrial fibrillation significantly reduced the increased platelet activation and reactivity associated with the procedure. In addition, rIPC has been reported to decrease thrombus formation in rats (Ropcke et al. 2012). Research findings become more valuable if successfully translated into clinical practice. Unfortunately, rIPC did not attain the desired benefit when applied in urological practice. Huang et al. (2013) have randomized patients undergoing laparoscopic partial nephrectomy into two groups with or without rIPC and have described the outcome in terms of changes in renal function by diuretic scintigraphy. The authors found that rIPC significantly affected the short-term renal function but this effect was not maintained on the long-term. These findings suggest studying the effect of rIPC on a larger scale and on different condition in order to optimize the outcome.

Conclusions

Remote ischemic preconditioning (rIPC) is a feasible and promising tool in alleviating renal I/R injury. The underlying pathophysiological mechanisms might include suppression of pro-inflammatory cytokines (*IL-1β, TNF-α* and *ICAM-1*) genes and enhanced expression of antioxidant (*Nrf2, HO-1* and *NQO-1*) genes and the anti-apoptotic (*Blc-2*) gene pathways.

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