

## The role of nitric oxide in the protective action of remote ischemic per-conditioning against ischemia/reperfusion-induced acute renal failure in rat

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### ABSTRACT

**Objective(s):** We investigated the role of nitric oxide (NO) in the protective effects of remote ischemic per-conditioning (rIPerC) on renal ischemia/reperfusion (I/R) injury in male rats.

**Materials and Methods:** I/R treatment consisted of 45 min bilateral renal artery ischemia and 24 hr reperfusion interval. rIPerC was performed using four cycles of 2 min occlusions of the left femoral artery and 3 min reperfusion at the beginning of renal ischemia. The animals were given normal saline (vehicle), NG-nitro-L-arginine methyl ester (L-NAME) or L-arginine. Following the reperfusion period, renal functional- and oxidative stress- parameters, as well as histopathological changes were assessed.

**Results:** In comparison with the sham group, I/R resulted in renal dysfunction, as indicated by significantly lower creatinine clearance and higher fractional excretion of sodium. This went along with decreased glutathione peroxidase (GPX) and catalase (CAT) activity in the I/R group, increased malondialdehyde (MDA) contents and histological damages. In comparison with the I/R group, the rIPerC group displayed improved renal function, increased activity of GPX and CAT enzymes, and decreased MDA level. However, these effects were abrogated by L-NAME injection and augmented by L-arginine treatment.

**Conclusion:** According to the results, the functional and structural consequences of rIPerC against I/R-induced kidney dysfunction, which is associated with reduction of lipid peroxidation and intensification of anti-oxidant systems, is partially dependent on NO production.

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### Introduction

Renal ischemia/reperfusion injury (IRI) is one of the serious reasons of acute renal failure, which is a prominent cause of mortality around the world. According to many documents, reactive oxygen species (ROS) have a significant role in the injury response to I/R. Increased generation of ROS and a decrease in cellular innate anti-oxidant accessibility lead to oxidant-induced damage in IRI.

One of the most potent innate mechanisms of cellular protection against IRI is remote ischemic per-conditioning (rIPerC). rIPerC involves application of short episodes of I/R instituted at a remote site during target organ ischemia (1), and is beneficial in unpredicted acute ischemic conditions (2). Although rIPerC was initially created to preserve myocardium against IRI in 2007 by Schmidt *et al.* (3), its protective effects have been thereafter shown in many non-cardiac limbs including kidneys (4, 5). But, it is still unclear how the protective signal translates from the remote organ to the kidney and which signaling pathways within the kidney are involved as a mediator of the protection. A recent evidence suggests that rIPerC reduces I/R-induced acute kidney injury partly by down-regulation

of inflammatory mediators (5). Besides, nitric oxide (NO) down-regulates inflammatory mediators (6). Remote limb ischemia results in washing of soluble mediators, which are suspected to be responsible for the protective effect, into the circulation. However, to date, there is no published report regarding the role of NO in the connections between remote and target organs.

In addition, a recent *in vivo* study has suggested that the benefits of rIPerC in the kidney results as a consequence of the reduction in the renal lipid peroxidation induced by I/R (7). But to date, there is no published report about the relationship of oxidative stress and NO during application of rIPerC technique to protect the kidney against IRI. However, several studies have implicated NO in protection induced by ischemic pre-conditioning (IPC). It has been reported that inducible nitric oxide synthase (iNOS) enzyme in the kidney is implicated in protection during the late phase of IPC induced by short episodes of I/R in mouse (8) and rat (9). Also, it has been shown that IPC leads to the activation of the iNOS isoform in the liver (10) and small intestine (11). NO inhibits the expression of adhesion molecules in the vascular endothelium leading to increased blood flow to ischemic regions (12). NO donors protect the kidney

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in the ischemic renal failure (13), and inhibition of NO synthesis increases susceptibility to kidney ischemia (14). By contrast, according to some investigations, inhibition of NOS protects organs against ischemia (15). Although these data suggest that NO has a role in the IPC- induced protection against a second exposure to I/R, the role of NO in the protective action of this novel strategy (i.e. rIPerC) against ischemic kidney injury has not yet been studied. Thus, in the present study we investigated the role of NO during rIPerC in a model of renal IRI in preventing renal dysfunction and improving sodium handling, and its possible relation to oxidative stress.

## Materials and Methods

All procedures were approved by the Ethics Committee for the Use of Experimental Animals at Shiraz University. Adult male Sprague-Dawley rats (230–300 g) were maintained under standard conditions (12 hr light–dark cycle; 20–22 °C) and had free access to water and standard rodent food.

### Experimental protocol

Rats were anesthetized using sodium pentobarbital (60 mg/kg; IP). Body temperature was monitored with a rectal thermometer and maintained at  $37 \pm 1$  °C. After a midline incision, renal artery and vein of both kidneys were carefully cleared and separated from each other. The rats were randomly assigned to seven groups (n = 10 in each group): (1) in sham group, renal arteries were not occluded; (2) in I/R group, rats were subjected to 45 min ischemia by bilateral clamping the renal artery; (3) in rIPerC group, rats were subjected to 45 min ischemia by bilateral clamping the renal artery and four cycles of 2 min of ischemia followed by 3 min of reperfusion of the left femoral artery beginning at the onset of renal ischemia; (4) in NG-nitro-L-arginine methyl ester (L-NAME) + I/R group, 20 mg/kg L-NAME was injected (IP) 30 min before ischemia; (5) in L-NAME + rIPerC group, 20 mg/kg L-NAME was injected (IP) 30 min before ischemia; (6) in L-arginine (L-Arg) + I/R group, 400 mg/kg L-Arg (as an NO substrate) was injected (IP) 30 min before ischemia; (7) in L-Arg + rIPerC group, 400 mg/kg L-Arg was injected (IP) 30 min before ischemia.

At the end of the surgery, rats were placed in metabolic cages and their urine was collected over a period of 24 hr. After 24 hr of reperfusion, rats were anesthetized and blood samples were taken from the inferior vena cava. Rats were killed (under anesthesia) and their kidneys were dissected on dry ice. Part of the kidney was fixed in 10% formalin for histological evaluation and the rest was immediately kept frozen at -70 °C.

### Renal functional assessments

The collected urine from the 24 hr reperfusion period was measured gravimetrically and total volume was recorded. Serum and urinary creatinine were measured by colorimetric methods (autoanalyser; Prestige, Biolis 24I, Japan) and were used in conjunction with urine flow to calculate creatinine clearance ( $C_{cr}$ ) as an indicator of glomerular function. Serum and urinary  $Na^+$  was measured at the end of the reperfusion period and used to estimate the fractional excretion of  $Na^+$  ( $FE_{Na}$ ) as an indicator of tubular dysfunction.

### Measurement of renal oxidative stress

#### Malondialdehyde levels

The renal tissue malondialdehyde (MDA) levels were determined in tissue samples by spectrophotometry (16). MDA reacts with thiobarbituric acid to produce a pink colour with a maximum absorbance at 532 nm.

#### Glutathione peroxidase activity

Glutathione peroxidase (GPX) activity was measured spectrophotometrically. The assay mixture consisted of tris buffer (pH 7.6), 3 mM NADPH, 20 mM cumene hydroperoxide, 40 mM reduced glutathione (GSH), and 10 U/ml GR. The activity was calculated using the molar extinction coefficient for NADPH ( $6.22 \mu\text{mol}^{-1} \times \text{cm}^{-1}$  at 340 nm).

#### Catalase activity

Catalase (CAT) activity was determined by Aebi's method (17). A decrease in absorbance at 240 nm was monitored for 1 min. The activity was calculated using the  $H_2O_2$  extinction coefficient ( $0.036 \mu\text{mol}^{-1} \times \text{cm}^{-1}$ ).

### Histological assessment

Renal tissue samples were fixed in the buffered 10% formaldehyde. After dehydration through a graded series of alcohol and clearing in xylol, the samples were embedded in paraffin and 5  $\mu\text{m}$  sections were stained with hematoxylin and eosin. In a blinded fashion, histopathology for each section was examined in at least 10 randomly selected non-overlapping fields under light microscope. The renal sections were evaluated for glomerular capillary lumen narrowing, reduction in the number of RBCs in glomerular capillary lumen, enlargement of Bowman's space, loss of brush borders, exfoliation of the proximal tubular cells into the lumen, dilatation of the lumen of blood vessels that are located between the tubules with congestion and increased number of RBC in the lumen (blood stasis in the veins). Sections were graded according to the changes involved, scoring 0 with no lesions; 1 with less than 20%, 2 with 20%–40%, 3 with 40%–60%, 4 with 60%–80%, 5 with greater than 80%. The sum of all numerical scores in each group was taken as the total histopathological score.

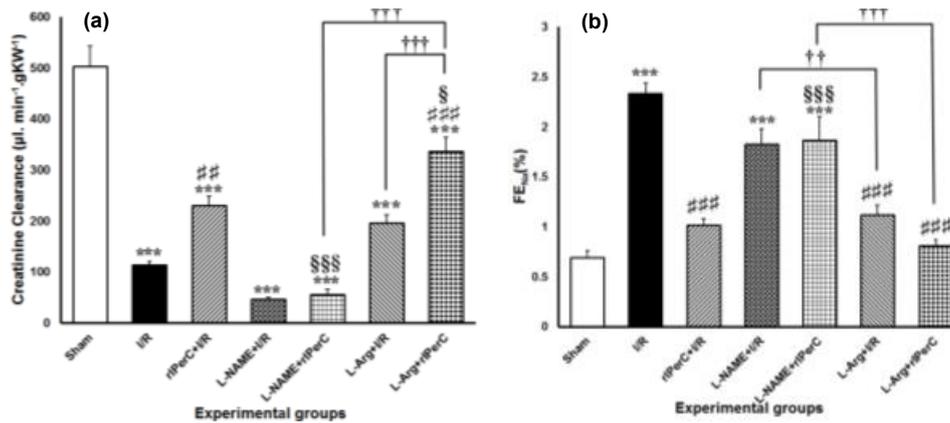
### Statistical analysis

All data (presented as mean  $\pm$  SEM) were analyzed with SPSS statistics software package (SPSS for Windows v. 22.0, Chicago, IL). To compare mean values across groups, a one-way ANOVA was performed with Tukey's *post-hoc* test. Significance was taken at  $P < 0.05$ .

## Results

### Effect of rIPerC on renal function

Renal I/R resulted in a significant decrease in  $C_{cr}$  and an increase in  $FE_{Na}$  in comparison with the sham group. Furthermore, rIPerC significantly improved  $C_{cr}$  and decreased  $FE_{Na}$  compared to the I/R group (Figure 1). Administration of L-NAME significantly reduced  $C_{cr}$  in I/R kidneys, which was unchanged when the rats were treated with rIPerC. L-Arg injection significantly ameliorated glomerular filtration rate (GFR) in I/R kidneys. It is remarkable that L-Arg treatment also ameliorated the  $C_{cr}$  in L-Arg + rIPerC treated rats. In



**Figure 1.** Effects of remote ischemic per-conditioning (rIPerC) on (a) creatinine clearance (CCr) and (b) fractional excretion of sodium ( $\text{FE}_{\text{Na}}$ ). Data are presented as mean $\pm$ SEM (n=10). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs sham group; # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.00, vs ischemia/reperfusion (I/R) group; § $P$ <0.05, §§ $P$ <0.01, §§§ $P$ <0.00, vs rIPerC group. † $P$ <0.05, †† $P$ <0.01, ††† $P$ <0.001, comparison between NG-nitro-L-arginine methyl ester (L-NAME) and L-Arginine (L-Arg)-received groups

addition, we found no change in  $\text{FE}_{\text{Na}}$  between rIPerC + I/R and I/R when NOS was inhibited by L-NAME. The injection of L-Arg clearly improved the  $\text{FE}_{\text{Na}}$  in both L-Arg + I/R and L-Arg + rIPerC groups vs. I/R group. These results clearly emphasize that NO possibly has a positive role in rIPerC-mediated functional protection against I/R-induced kidney injury.

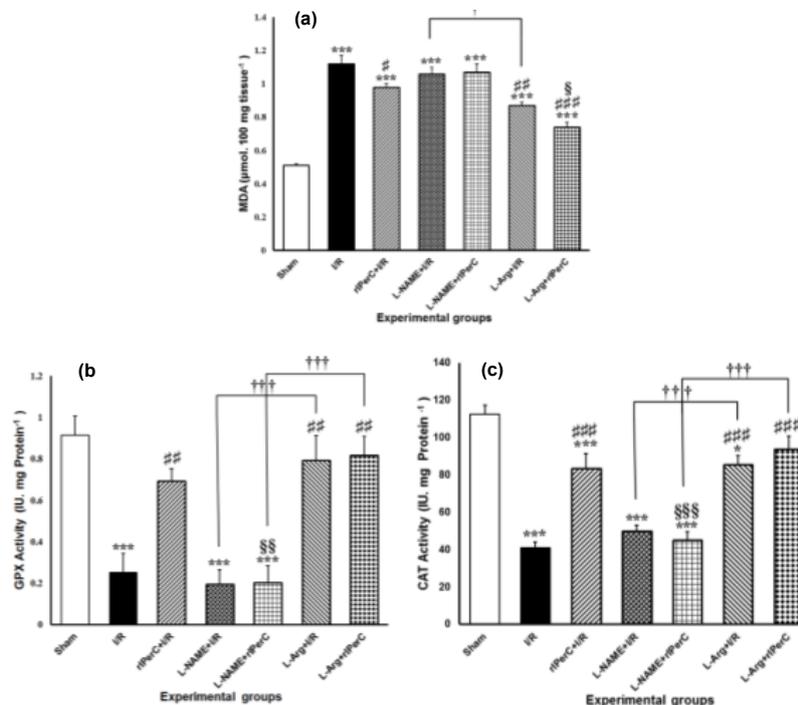
#### Effects of rIPerC on I/R-induced oxidative stress in the kidney

Significant increase in MDA content was observed in the I/R in comparison with sham group. However, rIPerC significantly decreased MDA content as compared to the I/R group (Figure 2a). Administration of L-NAME did not change MDA content in I/R kidneys, and also when

animals were treated with rIPerC. L-Arg administration significantly ameliorated MDA content in L-Arg + I/R and L-Arg + rIPerC kidneys (Figure 2a).

In addition, renal I/R significantly decreased GPX activity in comparison with the sham group. The GPX activity in the rIPerC group was significantly higher than that in the I/R group. Administration of L-NAME significantly reduced GPX activity in I/R kidneys and, similarly, in animals treated with rIPerC. L-Arg treatment significantly improved the GPX activity in I/R kidneys. L-Arg injection also improved the GPX activity in L-Arg + rIPerC group (Figure 2b).

Compared with the sham group, renal I/R induced significant reduction in CAT activity. The activity of CAT was increased in the rIPerC group compared with the I/R

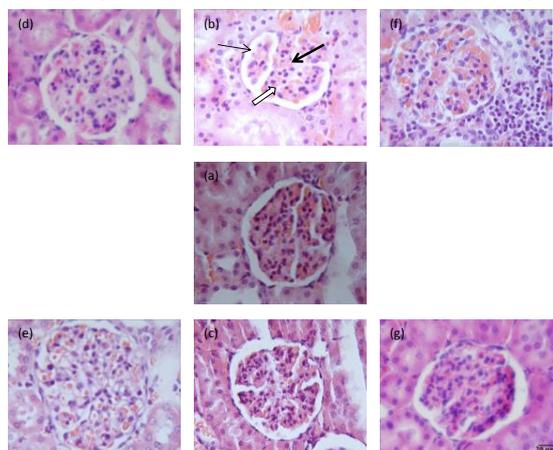


**Figure 2.** Effects of remote ischemic per-conditioning (rIPerC) on the renal tissue levels of (a) malondialdehyde (MDA), (b) glutathione peroxidase (GPX) and (c) catalase (CAT) activity. Data are presented as mean $\pm$ SEM (n = 10). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs sham group; # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.00, vs ischemia/reperfusion (I/R) group; § $P$ <0.05, §§ $P$ <0.01, §§§ $P$ <0.00, vs rIPerC group. † $P$ <0.05, †† $P$ <0.01, ††† $P$ <0.001, comparison between NG-nitro-L-arginine methyl ester (L-NAME) and L-Arginine (L-Arg)-received groups

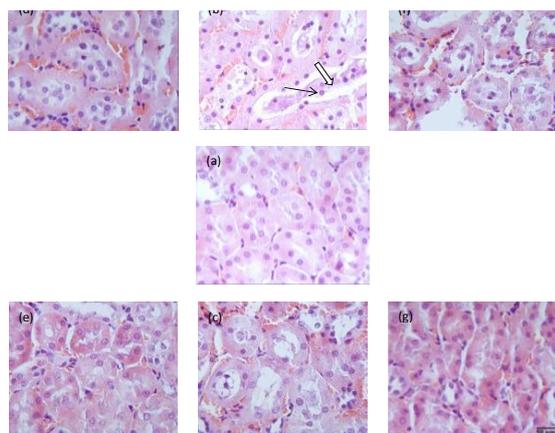
group. Administration of L-NAME significantly reduced CAT activity in I/R kidneys, which was unchanged in animals treated with rIPerC. In addition, L-Arg administration significantly improved CAT activity in L-Arg + I/R and L-Arg + rIPerC groups (Figure 2c).

### Effects of rIPerC on kidney histology

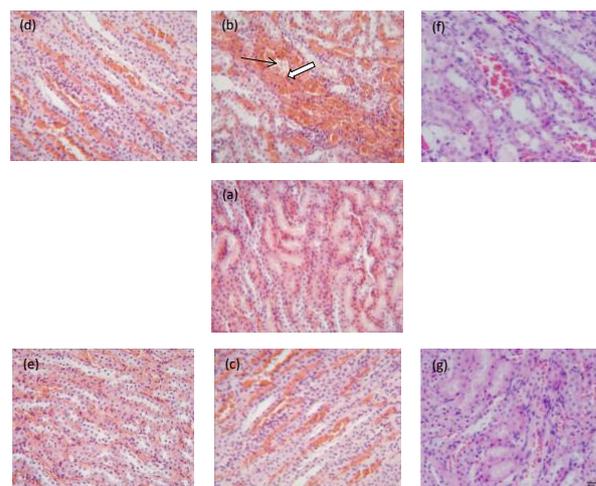
There was no histopathological change in the specimens from the sham group (Figure 3a, 4a, 5a). However, I/R resulted in major changes in renal tissues, including enlargement of Bowman's space, reduction in the size of glomerular tuft, glomerular capillary lumen narrowing, reduction of the number of RBC in glomerular capillary lumen, glomerular capillaries congestion (Figure 3b), loss of brush borders, exfoliation of the proximal tubular epithelial cells into the lumen of the tubules (Figure 4b) and medullary vascular congestion



**Figure 3.** Renal light microscopic images in the (a) sham (b) ischemia-reperfusion (I/R) and (c) remote ischemic per-conditioning (rIPerC), (d) NG-nitro-L-arginine methyl ester (L-NAME) + I/R (20 mg/Kg), (e) L-NAME + rIPerC (20 mg/Kg), (f) L-Arginine (L-Arg) + I/R (400 mg/Kg) and (g) L-Arg + rIPerC (400 mg/Kg) groups. Hematoxylin and eosin (H&E)-stained sections were evaluated for histological changes 24 hr after renal I/R injury (b) including enlargement of Bowman's space (thin arrow), reduction in the size of glomerular tuft (outlined white arrow), and reduction in the number of RBCs (thick arrow)



**Figure 4.** Renal light microscopic images in the (a) sham (b) ischemia-reperfusion (I/R) and (c) remote ischemic per-conditioning (rIPerC), (d) NG-nitro-L-arginine methyl ester (L-NAME) + I/R (20 mg/Kg), (e) L-NAME + rIPerC (20 mg/Kg), (f) L-Arginine (L-Arg) + I/R (400 mg/Kg) and (g) L-Arg + rIPerC (400 mg/Kg) groups. Hematoxylin and eosin (H&E)-stained sections were evaluated for histological changes 24 hr after renal I/R injury (b) including exfoliation of the proximal tubular cells into the lumen (thin arrow), and the loss of brush borders (outlined white arrow)



**Figure 5.** Renal light microscopic images in the (a) sham (b) ischemia-reperfusion (I/R) and (c) remote ischemic per-conditioning (rIPerC), (d) NG-nitro-L-arginine methyl ester (L-NAME) + I/R (20 mg/Kg), (e) L-NAME + rIPerC (20 mg/Kg), (f) L-Arginine (L-Arg) + I/R (400 mg/Kg) and (g) L-Arg + rIPerC (400 mg/Kg) groups. Hematoxylin and eosin (H&E)-stained sections were evaluated for histological changes 24 hr after renal I/R injury (b) including dilatation of the lumen of blood vessels that are located between the tubules with congestion (thin arrow) and increased number of RBC in the lumen (outlined white arrow)

**Table 1.** Total histopathological score in Sham, I/R, rIPerC + I/R, L/NAME + I/R, L-NAME + rIPerC, L-Arg + I/R, and L-Arg + rIPerC groups (each n=7) at the end of experiment (mean±SEM)

Group	Histopathological score
Sham	0.0 ± 0.00
I/R	38.1 ± 1.03***
rIPerC + I/R	19.7 ± 1.98****###
L-NAME + I/R	38.1 ± 1.03****§§§
L-NAME + rIPerC	28.5 ± 1.17****###§§§ψψψ
L-Arg + I/R	22.9 ± 1.28****###ψψψ
L-Arg + rIPerC	12.3 ± 1.78****###§§§ψψψ†††###

Values are expressed as mean±SEM

\*\*\* $P < 0.001$  Vs Sham group;  $\psi\psi\psi P < 0.001$  Vs L-NAME + I/R

### $P < 0.001$  Vs I/R; ††† $P < 0.001$  Vs L-NAME + rIPerC

§§§ $P < 0.001$ , §§ $P < 0.01$  Vs rIPerC + I/R; ††† $P < 0.001$  Vs L-Arg + I/R

(Figure 5b). However, rIPerC alleviated the I/R-induced changes in the kidney (Table 1). In the rIPerC group, there were less degrees of glomerular damages (Figure 3c), loss of brush border and exfoliation of the proximal tubular epithelial cells into the tubular lumen (Figure 4c) as well as medullary vascular congestion in comparison with I/R group (Figure 5c).

In the L-NAME + I/R group, enlargement of Bowman's space, reduction in the size of glomerular tuft, glomerular capillary lumen narrowing, reduction of the number of RBC in glomerular capillary lumen (Figure 3d), loss of brush borders, exfoliation of the proximal tubular epithelial cells into the lumen of the tubules (Figure 4d), and medullary vascular congestion (Figure 5d) were observed. Besides, L-NAME treatment diminished renal tissue protective effects of rIPerC strategy (Figure 3e, 4e, 5e). However, L-Arg treatment prevented the I/R-induced lesions in the kidney (Table 1), such that less

degrees of histopathological changes were observed in L-Arg + I/R group compared to I/R and L/NAME + I/R groups (Figure 3f, 4f, 5f). Furthermore, L-Arg treatment improved renal histopathological damages (Table 1) in the L-Arg + rIPerC group as compared to rIPerC + I/R group (Figure 3g, 4g, 5g).

## Discussion

In this study, rIPerC treatment suppressed renal oxidative stress, which was demonstrated by decreased MDA level and increased activities of anti-oxidant enzymes that was associated with favorable histological outcome. Moreover, rIPerC attenuated the I/R-induced renal dysfunction indicated by reduced GFR and increased fractional excretion of sodium. These beneficial effects of rIPerC were abrogated by L-NAME injection and augmented by L-Arg treatment. These results suggested that the renal tissue protecting effect of rIPerC is partly mediated by NO.

We measured  $C_{cr}$  as the index of GFR, which was significantly higher in rIPerC + I/R group compared with I/R group. It was accompanied with renal histological damages. However, rIPerC significantly attenuated the histological changes compared with those of I/R group, which exhibited enlargement of Bowman's space, reduction in the size of glomerular tuft, reduction of RBC number in glomerular capillary lumen, loss of brush borders, exfoliation of the epithelial cells into the lumen, and vascular congestion. These findings agree with and complement the findings of others (5).

NO production contributes to positive effects of ischemic pre-conditioning in the rat's heart (18), liver (19) and the kidney (8). In the kidney, however, it has not been elucidated whether the effect of rIPerC is mediated by the production of NO. In the current work, L-NAME treatment completely abolished the rIPerC-induced renal resistance against IRI, whereas L-Arg injection increased renal resistance to IRI. Thus, we propose that NO production is involved in the rIPerC-induced ischemic renal toleration. The action of NO on I/R kidney is considered as both cytoprotective (20) and cytotoxic (21). It seems that NO production is stimulated during rIPerC that exerts a protecting action.

NO is known to modify renal hemodynamic function by improving microcirculation (22). The I/R-induced severe reduction of RBCs in glomerular capillaries can be considered as an indicator of renal hypo-perfusion during the post-ischemic period. As the result of rIPerC treatment, RBC number was significantly decreased after L-NAME administration. Moreover, we observed an increase of RBC number in rIPerC and rIPerC + L-Arg groups. This observation is accordant with findings of others, who demonstrated that L-NAME prevented the restoration of renal blood flow (RBF) after reperfusion, whereas L-Arg restored RBF (23). However, another possibility could also be postulated in which rIPerC may induce some of the vasodilatory factors that can decrease renal vascular resistance to increase RBF. In current study, unlike L-Arg, L-NAME treatment abolished the rIPerC-induced elevation of RBC number; hence, we suggest that NO has an important role in rIPerC-induced increase of renal perfusion. Although it is possible that brief periods of ischemic stress stimulate the generation of NO over such a period, we had no tools of evaluating

NO level in our experimental model.

In renal IRI, the inflammatory response leads to endothelial activation and increased endothelial cell-leukocyte adhesion. The leukocyte-endothelial interaction impacts the outer medulla more than the cortex, displayed by the marked vascular congestion (24), as observed in the current research. Besides, ischemic injury results in the damage to the renal tubular epithelium, particularly to the S3 segment of the proximal tubule. Tubular injury leads to dysfunction, shedding of the cells, tubular obstruction and therefore to reduced kidney function, as was observed in this study. In the current work, I/R increased  $FE_{Na}$ , and total histopathological score, and reduced  $C_{cr}$ . Applying rIPerC significantly ameliorated kidney function and morphology as compared to the I/R group. In consistent with our results in the application of the rIPerC strategy, other investigators have shown that different forms of ischemic conditioning ameliorate kidney function and histology (25).

Overproduction of the ROS is increasingly implicated in renal IRI (26). An imbalance between ROS production and anti-oxidant defense system, including the GPX and CAT enzymes, leads to an increased oxidation of cellular macromolecules. In this study, CAT and GPX activities in the I/R group were significantly lower than the sham group. These observations agree with previous studies reporting that renal IRI is linked to decreases in GPX and CAT activities (27). The present study demonstrated that rIPerC prevents I/R-induced GPX and CAT depletion. Moreover, rIPerC was so effective in restoration of the activities of these anti-oxidants approximately to those of sham group. IPC protects the kidney against IRI via increases in the GPX and CAT activities (28). However, in our study L-NAME treatment abolished the rIPerC-induced elevation of GPX and CAT activity, whereas L-Arg restored it; so, we suggest that NO has an important role in rIPerC-induced increase in GPX and CAT activity. This result was in agreement with that of Adam (29), who found that IPC induced a significant increase in GPX activity in liver homogenate in comparison with the I/R group.

Several evidences suggest the possible link between oxidative stress and NO production. Oxidative stress involves the ROS-mediated oxidative degradation of the components of cellular membrane phospholipids followed by formation of peroxy radicals and finally lipid peroxides that are metabolized, via  $\beta$ -oxidation pathway, to MDA (30). ROS are produced by various sources including an impaired mitochondrial electron transport chain and NOS (31). NOS is involved in the development of renal IRI (32). Generation of ROS and NO in I/R injury leads to formation of cytotoxic metabolite and peroxynitrite, which can cause lipid peroxidation. Accordingly, tissue MDA level indicates lipid peroxidation. MDA has been implicated in renal IRI (33, 34). In the current work, I/R induced an increase in the renal MDA content that was associated with renal failure. On the other hand, rIPerC reduced renal MDA content. Renal histological improvement leads to a decrease in tissue oxidative injuries. In agreement with our results, other investigators found high lipid peroxidation after renal I/R injury and protecting effect of IPC against oxidative damage (35). However, in our

study L-NAME treatment diminished the rIPerC-induced attenuation of MDA content, whereas L-Arg restored it; therefore, we suggest that NO has an important role in rIPerC-induced decrease in lipid peroxidation. In line with this, Mahfoudh-Boussaid *et al.* (36) found that renal protective effect of IPC via reducing oxidative stress is associated with activation of NO production.

## Conclusion

Our research indicated that four cycles of 5 min-rIPerC partially protects against I/R-induced kidney injury by ameliorating hemodynamic disorders that are observed after reperfusion. This beneficial effect of rIPerC was abrogated by L-NAME administration and augmented by L-Arg injection. The results propose that the protective effects of rIPerC in the kidney may result from reduction of lipid peroxidation and augmentation of anti-oxidant systems that is partially mediated by NO. However, additional studies are needed to explore whether the renoprotective signal is due to NO production in the per-conditioned limb or in the kidney itself.

## Conflicts of Interest

The authors declare no competing financial interest.

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