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Scintigraphic evaluation of remote pre-conditioning protection against unilateral renal ischemia/reperfusion injury in rats: a longitudinal study

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Abstract

Purpose To determine the role of remote perconditioning (RPeC) on renal function and histology in an animal model of unilateral renal ischemia and reperfusion (IR) injury.

Methods Rats were subjected to 60 min unilateral renal ischemia. RPeC protocol was the application of four cycles of 5 min IR of left femoral artery during renal ischemia. Assessments of histological changes and renal function were made 24 h, 1 week, or 3 weeks later. 99mTc-DMSA scan was performed using a small-animals SPECT system.

Results 24-h reperfusion decreased the 99mTc-DMSA uptake in the left kidney compared to the intact kidney of control animals. RPeC group has higher uptake compared to the IR group. After 1 week and 3 weeks, uptakes were gradually increased in both groups and no differences were observed. Severe morphological changes in the ischemic kidneys of both groups were observed after 24 h which attenuated after 1 week and 3 weeks. Moreover, no differences in creatinine and BUN levels between IR-treated and intact animals were observed.

Conclusion These data suggest that RPeC exerts a partially transient improvement in the renal function in the first day after reperfusion. However, long-term follow-up study showed no beneficial effects of RPeC. Moreover, noninvasive 99mTc-DMSA scan revealed a suitable tool in the follow-up evaluation of recovery process in the unilateral renal IR injury models.

Keywords Ischemia/reperfusion injury · Acute kidney injury · Recovery process · Animal SPECT · Scintigraphy · Remote ischemic perconditioning · Follow-up study · Unilateral ischemia · Histopathology

Introduction

Ischemia/reperfusion (IR) injury which was characterized by restriction of blood supply to the kidneys followed by restoration of blood flow and reoxygenation is one of the main causes of acute kidney injury (AKI). AKI is a global public health issue and an important clinical syndrome with a high incidence of morbidity and mortality which has socio-economic burden due to lack of effective treatment [1, 2].

Remote ischemic conditioning (RIC) has emerged as a novel therapy for protecting different organs against IR injury [3, 4]. RIC describes the strategy in which cyclic sub-lethal ischemia and reperfusion applied to an organ or tissue distal to the target organ exerts protection against the detrimental effects of a prolonged lethal ischemia [5]. The RIC stimulus can be induced non-invasively to the limb using a standard blood pressure cuff and can be applied either prior to (remote ischemic preconditioning, RPC) or after renal reperfusion (remote ischemic postconditioning, RPoC).

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Although RPC and RPoC have been shown to reduce post-ischemic injury in many organs, these two methods have their limitation. For example, application of RPC requires foreknowledge of the ischemic event, which is difficult to anticipate in most circumstances, and RPoC is more applicable to the clinical scenarios with manual reperfusion.

The newly described models of RIC is remote ischemic perconditioning (RPeC) which firstly reported in 2011 by Kadkhodaee and colleagues to attenuate AKI induced by unilateral renal ischemia with nephrectomy in the acute phase (24 h) of reperfusion [6]. In this model, sublethal cyclic IR of the limb is applied during renal ischemia as RPeC protocol. This model may have benefit in the case of unpredicted kidney ischemic insult. However, no studies have been established to assess the benefit determined by RPeC after a longer period of reperfusion, such as 7–21 days. Moreover, the effect of RPeC in unilateral renal ischemia without nephrectomy is not reported yet.

Scintigraphy is a noninvasive screening test which is specifically used to evaluate the mass of functional renal tissue. Renal radionuclide scan has been used for assessment of renal function in animal studies [7, 8]. This assay provides a quantitative measurement with more accurate information about the functional status of kidneys during the time in a single animal. In this study, we used technetium-99m dimercaptosuccinic acid (99mTc-DMSA) quantitative scan to assess the protective effect of RPeC on restoration capacities of renal tubular cells after experimental induction of unilateral renal IR injury during a follow-up of 21 days.

Materials and methods

Animals and surgical procedure

All procedures were approved by the Animal Ethic Committee at the Bushehr University of Medical Sciences (ethic code: BPUMS.REC.1394.14) which were in accordance with the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011, published by The National Academies Press). Male Wistar rats weighting 200–250 g were fed a standard diet and allowed free access to water. All surgeries were conducted under deep anesthesia with a combination of ketamine 100 mg/kg and xylazine 10 mg/kg (intraperitoneal injection). Once anesthetized, the rats were placed on a heating pad device and under a heating lamp to maintain a 37 °C body temperature throughout surgery. The left unilateral ischemic renal injury was induced by exposing the kidney through a flank incision and then applying a bulldog clamp across the renal pedicle. The right kidney was left intact. Another group of animals were subjected to a similar surgical procedure with same ischemic time plus receiving RPeC protocol as a therapeutic intervention. At the end of renal ischemia, the clamp was removed, global reperfusion was confirmed by loss of kidney cyanosis, and then the incision was sutured in two layers.

Remote perconditioning protocol

The protocol of RPeC was four cycles of 5-min left femoral artery occlusion and 5 min of reperfusion applied during renal ischemia. Preparation of left femoral artery was performed before the beginning of renal ischemia through a small skin incision in the left thigh. After that, the femoral neurovascular bundle was exposed and the femoral artery separated from the femoral nerve and vein at the proximal site near the groin. Ischemia and reperfusion of left femoral artery with an atraumatic microvessel clamp was achieved for induction of RPeC.

Post-ischemic time course experiments

At either 24 h, 1 week, or 3 weeks post-induction of renal ischemia, the rats were weighed and reanesthetized with ketamine and xylazine. The plasma samples were taken from the inferior vena cava for subsequent creatinine and blood urea nitrogen (BUN). Both kidneys were excised through a midline abdominal incision. Then, post-ischemic and contralateral kidneys were immediately fixed in 10% formalin. Two other groups of animals received the same protocols of IR and RPeC and used for scintigraphic evaluation of renal recovery during 3 weeks. The results from the post-ischemic and contralateral (uninjured) kidneys of the animals in each time point of reperfusion were contrasted against each other and against values observed in the kidneys from normal rats.

Radionuclide scintigraphy

Rats were anesthetized with a combination of ketamine and xylazine and were placed between the heads of the HiReSPECT small-animal SPECT (Parto Negar Persia Co., Iran) [8, 9]. The HiReSPECT is a dual-headed gamma camera equipped with parallel-hole collimators and pixelated CsI (Na) crystal containing 38×80 pixels (1 mm × 1 mm). A DMSA kit (Pars isotope, Iran) was prepared according to the manufacturer's recommendations. Renal scintigraphy performed after injection of 3 mCi of 99mTc-DMSA through the tail vein using insulin syringe. The results of our previous work showed that, the optimized image acquisition time for 99mTc-DMSA renal scan in rat is 1–2 h post-injection [8]. Hence, 1 h after radionuclide injection, a pair of two-dimensional opposed images (ventral and dorsal) for each rat were collected in a 38×80 matrix.

Image analysis

The relative uptake of 99mTc-DMSA by kidneys calculated after the selection of a region of interest (ROI) around the kidneys and background areas on both of the anterior and posterior images. The distribution of the activity in the kidneys was determined by measuring the photon counts within the selected ROI. The average of the activity uptake in the kidneys was obtained from the geometric mean of both of the ventral and dorsal images after background correction.

Histological assessment of renal injury

Renal tissue specimens, i.e., IR-damaged left kidneys and contralateral non-ischemic kidneys, were taken for histological analysis. Kidney tissue was fixed in 10% formalin, sectioned and stained with hematoxylin and eosin (H&E). Histopathological changes were assessed on 4-µm-thick sections by scoring tubular cell necrosis, tubular dilatation and brush border loss cast formation and congestion in 12 non-overlapping fields (40× magnification) of the cortex and corticomedullary junction. Tissue damages were scored according to the severity of lesions on a semi-quantitative scale, where 0, minimal or no lesion; 1, lesion involving < 25% of the field; 2, lesion involving 25–50% of the field; 3, lesion involving 50–75% of the field; and 4, lesion involving > 75% of the field.

Statistical analysis

Results are presented as mean ± SEM. Two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test was used. A paired *t* test was used to test for significant differences between measured at different time intervals within each experimental group. A probability value of $P < 0.05$ was accepted to be statistically significant.

Results

Effect of RPeC on 99mTc-DMSA absorption in unilateral IR-induced renal injury

As depicted in Fig. 1, 60-min unilateral left renal pedicle occlusion and 24-h reperfusion decreased 99mTc-DMSA uptake in the left kidney compared to the intact kidney of control animals. Table 1 shows the relative renal uptake of 99mTc-DMSA and counts/pixel values for each group. The evaluation of results indicates that the rats receiving RPeC protocol have higher relative renal Tc-99m DMSA uptake compared to the non-treated IR group in 24 h after reperfusion and it was accompanied by increased background activity. After 1 week of reperfusion, 99mTc-DMSA uptake increased in both groups. However, this was more pronounced for the IR group. After 3 weeks, 99mTc-DMSA increased in both groups compared to 1 week and this improvement was the same in both IR and RPeC groups. Moreover, background activity was very low after 3 weeks.

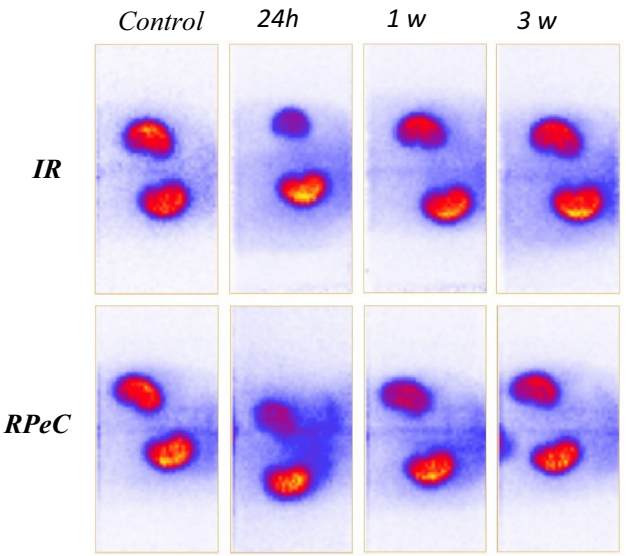

Fig. 1 Tc-99m DMSA uptakes at different time points after reperfusion in rats. The uptake is decreased and background activity is increased in the left kidney of rat in IR group, compared to the left kidney of the rat receiving RPeC protocol in 24 h. While after 1 week and 3 weeks, Tc-*99m* DMSA uptake was lower than the left kidney of rat in IR group, compared to the rat in RPeC group. Background activity was very low after 3 weeks ($n = 2$)

Table 1 The results of tissue Tc-99m DMSA uptake in control animals, and IR and RPeC groups at different time points of reperfusion ($n = 2$)

Groups	Time	Tc-99m DMSA uptake (LC/(RC + LC)) (%)	Tc-99m DMSA uptake (count/pixel)
Control		44.58 ± 2.25	510 ± 53
IR	24 h	31.86 ± 5.56	419 ± 61
	1 week	42.38 ± 1.61	572 ± 29
	3 weeks	45.53 ± 0.43	614 ± 39
RPeC	24 h	34.75 ± 1.83	410 ± 121
	1 week	39.77 ± 3.46	499 ± 65
	3 weeks	45.35 ± 5.56	603 ± 105

Results were expressed as mean ± standard deviation

LC left kidney count, *RC* right kidney count

Effect of RPeC on BUN and creatinine in unilateral IR-induced renal injury

As depicted in Fig. 2a, creatinine of 0.65 ± 0.06, 0.60 ± 0.05, and 0.60 ± 0.03 mg/dl were observed at 24 h, 1 week, and

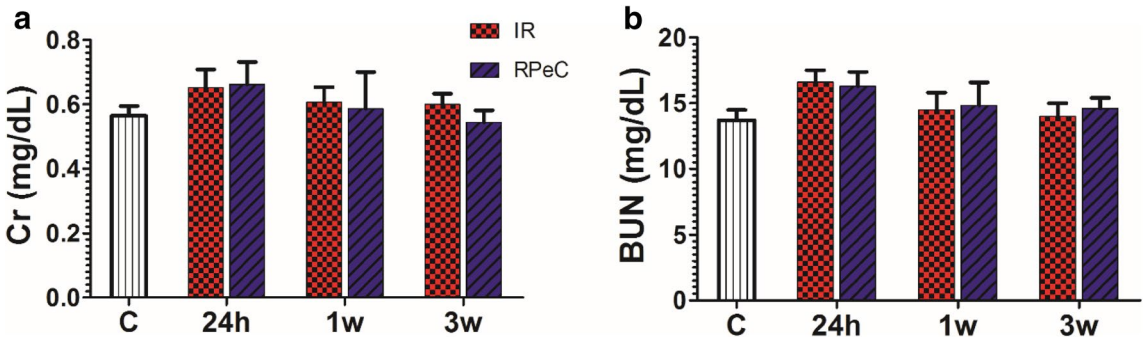

Fig. 2 Renal function at different time points after reperfusion in rats. *C* control, *IR* ischemia and reperfusion, *RPeC* remote perconditioning, *Cr* creatinine and BUN, blood urea nitrogen. There was no significant difference in the Cr (**a**) and BUN (**b**) levels between groups at different times of reperfusion ($n=7$)

3 weeks post-ischemia (respectively), in the IR group which has shown no significant difference in creatinine levels of intact control animals (0.56 ± 0.03 mg/dl). Also, the results of creatinine in the RPeC were not significantly different from those of IR group and control levels at each time point (0.66 ± 0.07, 0.59 ± 0.11, and 0.54 ± 0.04 mg/dl at 24 h, 1 week, and 3 weeks post-ischemia, respectively).

Also, BUN levels in the IR and RPeC groups showed no significant differences with those of normal rats (13.71 ± 0.78 mg/dl) at each time point (Fig. 2b). It was 16.60 ± 0.93, 14.50 ± 1.31, and 14.00 ± 1.00 mg/dl in the IR group and 16.28 ± 1.11, 14.83 ± 1.75, and 14.60 ± 0.81 mg/dl in the RPeC group at 1 day, 1 week, and 3 weeks post-ischemia (respectively).

Effect of RPeC on histopathological changes in unilateral IR-induced renal injury

According to the Histopathological scores (Table 2) the most prominent histological changes in the sections of IR group at 1-day post-ischemia were widespread proximal tubular necrosis, most notable in the outer medullary stripe (Fig. $3b_1$, vs. normal contralateral sections depicted in Fig. 3a). Tubular debris was sloughed into the lumina and apparent with resulted cast accumulation. Furthermore, overt tubular dilatation and loss of brush borders were predominant. Moreover, there were significant congestions of the inner cortex with erythrocytes. In the sections of RPeC-treated animals, similar changes were observed at 24 h and there was no significant difference between two groups.

By 1 week, there was extensive tubular dropout and considerable cellular expansion of the interstitial space (Fig. $3b_2$). Cast formation, tubular necrosis, and congestion were decreased in both IR and RPeC groups compared with 24 h. However, no significant difference was observed between two groups at this time point. After 3 weeks, decreased tubular necrosis and no tubular cast were observed in both groups with no significant difference between groups. However, the congestion in the IR group was more attenuated after 3 weeks compared with RPeC group. Of note, the contralateral kidneys maintained normal histology (no apparent histologic differences from the kidneys of normal rat), signifying that the damaged kidney did not cause overt contralateral kidney injury.

Discussion

Renal cortical scintigraphy using 99mTc-DMSA is being suggested as the favored technique for evaluation of renal functional recovery following several kinds of kidney insults

Table 2 Histopathological score

Time of reperfusion	Tubular necrosis		Cast formation		Tubular dilatation		Congestion	
	IR	RPeC	IR	RPeC	IR	RPeC	IR	RPeC
24 h	3.00 ± 0.27	3.25 ± 0.25	2.83 ± 0.42	2.4 ± 0.31	3.83 ± 0.11	3.75 ± 0.31	2.42 ± 0.26	2.83 ± 0.47
1 week	1.33 ± 0.31	1.33 ± 0.26	0.50 ± 0.19	0.17 ± 0.11	3.58 ± 0.19	3.58 ± 0.23	0.83 ± 0.21	0.83 ± 0.30
3 weeks	0.42 ± 0.15	0.58 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	3.25 ± 0.37	3.5 ± 0.23	0.25 ± 0.13	1.33 ± 0.31

Ischemia and RPeC kidneys were examined microscopically at each time point and graded for tubular necrosis, tubular dilatation and loss of brush borders, cast formation, and congestion. The mean ± SEM of the score of all fields examined per time point is presented ($n=4$)

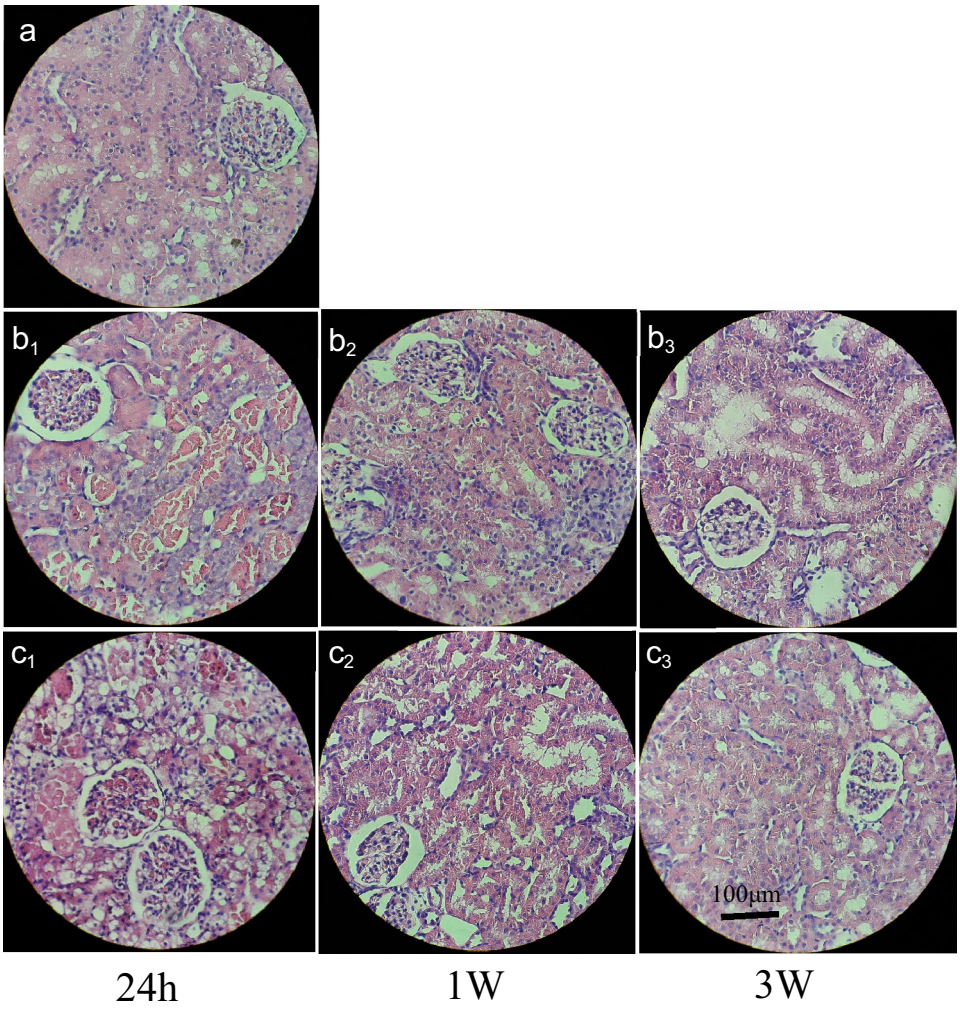

Fig. 3 Renal histologic hematoxylin and eosin (H&E) sections from normal kidneys, 24 h, 1 week and 3 weeks post-ischemia. **a** Normal kidney section demonstrating histology of renal cortex; $\mathbf{b_1}$–$\mathbf{b_3}$ post-ischemic kidney section of the IR group; $\mathbf{c_1}$–$\mathbf{c_3}$ post-ischemic kidney section of the RPeC group at different time points of reperfusion ($n=4$)

[10, 11]. The advantages and disadvantages of imaging methods for medical practice were discussed by Majd et al., and the value of 99m Tc-DMSA, in the age of high technical capabilities of MRI, CT, and ultrasound, method was emphasized [12]. It has been proved or established that the uptake of 99mTc-DMSA correlates with glomerular filtration rate, effective renal plasma flow, and creatinine clearance. Its quantitative measurement is, therefore, a good index for renal function. Previous studies have shown that 99m Tc-DMSA uptake differentiates normal from diseased kidneys [13].

Therefore, the objective of the present study was follow-up evaluation of the protective effect of RPeC on restoration capability of renal tubular cells, and furthermore to perceive its reliability as an indicator of kidney repairing capacity in renal IR injury. Quantitative 99mTc-DMSA renal uptake was assessed in RPeC-treated and IR-non-treated animals after unilateral renal ischemia at different time points of reperfusion. The improved renal function indicated by higher uptake of 99mTc-DMSA at 24 h in the ischemic left kidney of RPeC-treated animals compared with those of the IR-non-treated rats. This is in line with our preceding results as well as other laboratory study which indicating the protective effects of RPeC on histological changes and classical renal functional indices such as BUN and creatinine in the animal models of renal IR injury [14, 15].

Our previous studies suggested the involvement of neural signaling pathway and modulated pro-inflammatory cytokine in the protection afforded by RPeC in the right nephrectomy/45 min left ischemic kidney model of acute kidney injury [16]. Moreover, the protective effects of RPeC on the liver as a remote organ from the ischemic kidney is reported [17]. Accordingly, the study by Kristensen et al. suggested that RPeC might prevent dysregulation of renal salt and water handling via regulation of aquaporins phosphorylation and expression as well as via regulation of Na–K-ATPase in the post-ischemic rat kidneys [14]. Recently, Gholampour et al. reported that RPeC has protective effects on the kidney after 45-min renal ischemia and 24 h reperfusion, which might be related with augmentation of anti-oxidant systems and inhibition of TLR4 signaling pathway [18]. Moreover, RPeC has been shown to protect the other organs from IR

injury including the heart [19], brain [20] and liver [21] in both experimental and clinical studies. However, no study has reported the long-time effect of RPeC.

The follow-up study of the animals in 1 week and 3 weeks of reperfusion showed that after 1 week of reperfusion, 99mTc-DMSA uptake was increased in both groups, which indicated the improvement of renal function in both RPeC-treated and IR-untreated animals compared with 24 h. However, it was a little more pronounced for the IR group. After 3 weeks, 99mTc-DMSA uptake was increased in both groups compared to 1 week and this improvement was the same in both IR and RPeC groups. Moreover, background activity was very low after 3 weeks which indicates the increment of the kidney function which leads to increment of the radionuclide uptake. The protection offered by RPeC seems transitory because no difference was verified after 3 weeks between these two groups and renal function was close to the baseline values at day 21. This is in line with earlier studies reporting transient benefit effects of ischemic conditioning or no significant long-time protection by this intervention. A systematic review and meta-analysis by Hu et al. indicated that AKI in the RPC group was 11.5%, significantly less than the 23.3% incidence in the control group. Moreover, their results suggested that RPC had no significant effect on the occurrence of stages 1–3 acute kidney injury or renal replacement therapy, change in serum creatinine and estimated glomerular filtration rate (eGFR), hospital or 30-day mortality, or length of hospital stay [22].

Consistent with 99mTc-DMSA uptake data, most severe histopathological alterations were observed in the ischemic kidneys specimens of IR group which is in line with the results of previous works [23, 24]. However, the histopathological results of RPeC-treated animal showed no significant difference compared with the IR group. After 1 week and 3 weeks, renal histological changes showed significant recovery in both groups which is in good accordance with scintigraphy results. However, some histopathological alteration such as tubular dilation and loss of brush border were remained in both group after 3 weeks.

The reno-protection of RPeC in the present experiment was not as effective as findings of our previous studies [6, 15]. This result may be attributable to a prolonged ischemic time (60 min vs 45 min in our previous reports) and/or a unilateral renal IR model used for valuation of this therapeutic intervention, limb RPeR (compared to the right nephrectomized/left ischemic model in the previous studies). It is well accepted that at least two factors determine the ultimate severity of the subsequent renal injury: the length of the initial ischemia, and the existence of a normal contralateral kidney. The latter was suggested to worsen contralateral ischemic kidney damage by two mechanisms: (1) the existence of a normal intact kidney exaggerated instant post-ischemic renal vasoconstriction, potentially extending the ischemic insult; (2) by dropping glomerular filtration rate, the vasoconstriction deteriorates cast formation by attenuating renal tubular debris washout. Moreover, it is well accepted that the response of ischemic tissue to therapeutic intervention depends on the degree of initial tissue damages. Thus, when regarding these features together, it could be assumed that prolonged ischemic time and existence of intact contralateral kidney in the animal model of the present study, worsen the kidney damages which resulted in attenuated protection induced by RPeC compared with our previous reports.

The results of the current study indicate that RPeC exerts a partially transient improvement in the ability of rat kidneys to tolerate subsequent ischemic damage in the first days after 60-min unilateral renal ischemia. However, long-term follow-up study showed no beneficial effects of RPeC. We suggest examining the effect of RPeC on the shorter unilateral ischemia time and conduct the studies in a larger animal model such as pigs or dogs with a longitudinal long-term follow-up which are required to assess the protective effect of RPeC on the functional improvement in kidneys exposed to IR. Furthermore, noninvasive 99mTc-DMSA scan showed to be a valuable tool for the studies in the rodent model of unilateral kidney IR injury.

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Compliance with ethical standards

Conflict of interest All authors have no conflict of interest.

Ethical approval All procedures of this study were approved by the Animal Ethic Committee at the Bushehr University of Medical Sciences (ethic code: BPUMS.REC.1394.14) which were in accordance with the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011, published by The National Academies Press).

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