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Protective effects of limb remote ischemic per-conditioning on the heart injury induced by renal ischemic-reperfusion through the interaction of the apelin with the RAS/iNOS pathway

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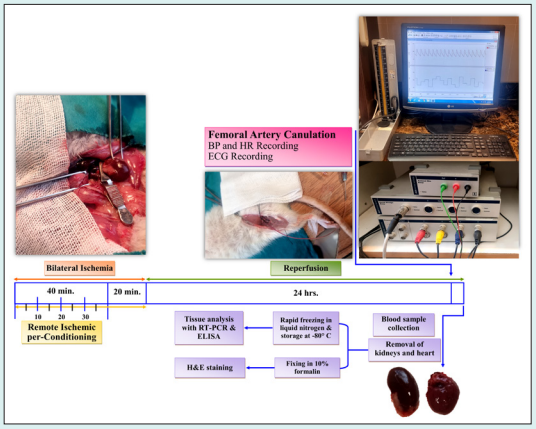
Abstract

Introduction: Remote ischemic conditioning upregulates endogenous protective pathways in response to ischemia-reperfusion injury. This study tested the hypothesis that limb remote ischemic per-conditioning (RIPerC) exerts cardioprotective effects via the renin-angiotensin system (RAS)/ inducible nitric oxide synthase (iNOS)/ apelin pathway.

Methods: Renal ischemia-reperfusion injury (I/R) was induced by bilateral occlusion of the renal pedicles for 60 minutes, followed by 24 hours of reperfusion; sham-operated rats served as controls. RIPerC was induced by four cycles (5 minutes) of limb ischemia-reperfusion along with bilateral renal ischemia. The functional disturbance was evaluated by renal (BUN and creatinine) and cardiac (troponin I and lactate dehydrogenase) injury biomarkers.



Results: Renal I/R injury increased renal and cardiac injury biomarkers that were reduced in the RIPerC group. Histopathological findings of the kidney and heart were also suggestive of amelioration injury-induced changes in the RIPerC group. Assessment of cardiac electrophysiology revealed that RIPerC ameliorated the decline in P wave duration without significantly affecting other cardiac electrophysiological changes. Further, renal I/R injury increased the plasma (322.40±34.01 IU/L), renal (8.27±1.10 mIU/mg of Protein), and cardiac (68.28±10.28 mIU/mg of protein) angiotensin-converting enzyme (ACE) activities in association with elevations in the plasma and urine nitrite (25.47±2.01 & 16.62±3.05 µmol/L) and nitrate (15.47±1.33 & 5.01±0.96 µmol/L) levels; these changes were reversed by RIPerC. Further, renal ischemia-reperfusion injury significantly (P=0.047) decreased the renal (but not cardiac) apelin mRNA expression, while renal and cardiac ACE2 (P<0.05) and iNOS (P=0.043) mRNA expressions were significantly increased compared to the sham group; these effects were largely reversed by RIPerC.

Conclusion: Our results indicated that RIPerC protects the heart against renal ischemia-reperfusion injury, likely via interaction of the apelin with the RAS/iNOS pathway.

Introduction

Renal ischemia-reperfusion (I/R) is a major cause of acute kidney injury (AKI) that is usually induced in some surgeries (kidney transplantation and coronary artery bypass grafting) and multiple clinical situations, including trauma, severe infection, sepsis, and hemorrhagic shock.[1,2] Renal I/R is characterized by sudden restriction of blood supply to the kidney followed by the subsequent restoration of perfusion and re-oxygenation.[3,4]

The consequences of renal I/R injury are local as well as remote organ destruction such as liver,[5] lung,[6,7] and heart.[8] The physiological and pathophysiological

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interaction between the kidney and the cardiovascular system provides essential indicators for maintaining homeostasis.[9] Indeed, several studies have demonstrated cardiac dysfunction contributes to a high morbidity/mortality rate after renal I/R injury.[10] It is often followed by a change in renin-angiotensin system (RAS) components and redox imbalance that induces renal and extra-renal organ disturbances.[11,12]

Oxidative stress, as one of the direct effects of the hypoxia process, is further developed during reperfusion.[11] Activity of angiotensin-converting enzyme (ACE), which can increase the concentration of angiotensin II (Ang II) in the plasma by cleaving angiotensin I (Ang I), was shown to be significantly increased in renal and cardiac after renal I/R injury.[13] The excess Ang II can intensify renal ischemia and promote renal and heart injury by increasing systemic inflammation, reactive oxygen species formation, altering the source of nitric oxide (NO) availability, and inducing nitrosative and oxidative stress.[14,15] ACE2, the first known homolog of ACE, was indicated to be a negative regulator of RAS because of its ability to convert Ang II to Ang (1–7), a peptide with vasodilatory and anti-fibrotic effects.[16]

One of the newly-recognized endogenous peptides that has attracted researchers' attention in the last decade is apelin. Apelin is an inotropic and cardioprotective ligand that shows positive effects by activating the G-protein-coupled receptor (APJ) in the pathology of cardiovascular diseases.[17] Apelin receptor has about 50% homology with angiotensin II type 1 (AT1) receptor. However, a counter-regulatory role for apelin in relation to the renin-angiotensin system is suggested by antagonizing the effects of angiotensin II on vascular tone, blood pressure, and fluid homeostasis.[18] Therefore, there are direct cellular and biological interactions between these two systems. On the other hand, apelin exhibits renal and cardioprotective effects through modulation of the nitric oxide synthesis (NOS)/NO-generation pathway.[19]

Hormesis or conditioning is a technique by which harmful stimuli below the threshold of injury are applied to an organ or system to develop cellular tolerance against more severe damage.[20] Moreover, it was demonstrated that conditioning could be done in a distant (remote) non-vital organ, such as a limb, but still exert beneficial effects on vital organs. Remote ischemic per-conditioning (RIPerC) is an excellent example of episodes of occlusion and reperfusion of blood flow on a limb, making temporary limb ischemia and remotely promoting endogenous protective pathways in the heart and kidney or other organs.[21,22]

The exact endogenous mechanisms of RIPerC are not completely understood yet. However, signal transmission from the remote site to target organs is reported to be modulated by humoral factors, immune cells, and the autonomic nervous system.[22] Therefore, this study tested the hypothesis that limb remote ischemic per-conditioning (RIPerC) exerts cardioprotective effects via the RAS/inducible nitric oxide synthase (iNOS)/apelin pathway.

Materials and Methods

Animals

This study was done on 30 male Sprague-Dawley rats weighing 250-270 g. They were purchased from the Animal Center of Shiraz University of Medical Sciences. Animal work took place in a specific animal room (in separate cages) at a controlled temperature of 22 to 25 °C with lighting (12 hours light/dark cycles) and was acclimatized to water and food without restriction.

Surgical procedure

Rats were anesthetized with Ketamine (50 mg/kg; Alfasan, Woerden, the Netherlands) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany) and were then put on a surgical heating table to retain the body temperature at 37 °C. The procedure of renal ischemia is described in our previous study.[23] Briefly, a midline abdominal incision was made, and the renal pedicles were exposed. Rats were randomly allocated to three groups (n=10 per group): (1) Sham, in which renal pedicels did not clamp; (2) BIR (bilateral ischemic-reperfusion) group, in which both renal pedicles were occluded for 60 minutes using microaneurysm vascular clamp; (3) BIR+RIPerC (remote ischemic per-conditioning) groups, in which four cycles of 5 minutes ischemia of the left femoral artery followed by 5 minutes reperfusion were applied at the beginning of renal ischemia. After the termination of ischemia, the clamps were token up and confirmed for appropriate reperfusion of the blood flow to the ischemic kidney. The abdominal incision was then sutured by 2/0 stitches in 2 layers.

Experimental protocol

At the end of the surgery, the animals were kept in metabolic cages as previously described,[24] and 24 hours urine volume was collected. After 24 hours reperfusion, all rats were weighed and immediately re-anesthetized. Animals were placed on a wooden plate to prevent additional electrical signals which could interfere with our ECG recording. Then, a filled cannula [normal saline (SAMEN, Mashhad, Iran) with 15 IU/mL heparin (Alborzdarouco, Tehran, Iran)] was inserted into the right femoral artery and connected to a pressure transducer. ECG electrodes were connected to the hands of animals (negative: right hand and positive: left hand) and the left limb. After 15 minutes of equilibration, arterial blood pressure (BP) and heart rate (HR) were continuously recorded for 10 minutes using a PowerLab/8SP data acquisition system (AD Instruments, BellaVista, NSW, Australia). Blood samples were taken from the abdominal vein to measure renal and cardiac function markers. Subsequently, the kidneys and heart were removed and weighed immediately. Two coronal sections were cut from the middle part of the kidney and heart. The left kidney and one piece of heart tissue were stored at -80 °C for

measuring ACE activity, NO metabolites, and RT-PCR. The right kidney and another slice of the heart were kept in 10% formalin for hematoxylin-eosin (H & E) staining.

Measurement of renal and cardiac functional biomarkers

Plasma concentrations of creatinine (Cr), blood urea nitrogen (BUN), and lactate dehydrogenase (LDH) were measured using Technicon® RA-1000 auto-analyzer (Technicon Corporation, Tarry-town, USA) in Namazi Hospital Laboratory, Shiraz, Iran. Troponin I level is a specific index of myocardium injury. Cardiac troponin I (cTn-I) was measured using an enzyme-linked fluorescent assay (ELFA) kit (VIDAS®, bioMérieux, North Carolina, USA).

Echocardiographic (ECG) analysis

ECG long lead II was recorded while the rats were lying on a wooden plate to avoid additional electrical signals which could interfere with our recording. Intervals of R-R and J-T (distance between S wave and the end of T wave), durations and amplitudes of P, Q, R, S as well as T waves, and the heart rate were analyzed by ECG analyzing software of a Power Lab system (AD Instruments, Australia). QT interval was corrected using normalized Bazett's equation QTc= QT/(RR/f)1/2, where f is the normalization factor according to RR duration in the sham group.[25]

ACE activity assay

The ACE activity was measured, as previously described by Beneteau et al with minor modifications.[26] In brief, ACE activity was determined with an artificial substrate [furanacryloyl-L-phenylalanylglycylglycine (FAPGG, Sigma-Aldrich, St. Louis, Missouri, United States)] in a reaction mixture containing 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES, Sigma-Aldrich], 0.5 mM FAPGG, 300 mM NaCl (Sodium chloride, Sigma-Aldrich), and the desired dilution of the serum or tissue homogenate at pH 8.2. The reaction rate at 340 nm can be determined using the FAPGG extinction coefficient (ε=0.989 μM^{-1}). Measurements were performed in 96-well plates at 25 °C. Changes in the optical density (340 nm) were measured at 1 min intervals for 10 min with a microplate reader (Epoch 2, BioTek, Winooski, Vermont, United States). One unit of ACE activity is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of FAPGG to FAP per minute at 25 °C. The ACE activity in the serum and tissue samples was expressed as µmol of FAPGG oxidized/min/mL of the serum or mg of protein (Units/mL of serum or Units/mg of protein).

Measurement of NO metabolite

The amount of NO can be measured by determining the concentrations of nitrite and nitrate end products in the plasma and tissue samples by the Griess method.[27] Initially, plasma samples were deproteinized using 99% ethanol and centrifuged. Quantitation of nitrite was based on its reaction with a mixture of 0.1% NEDD [N-(1-Naphthyl)ethylenediamine, Sigma-Aldrich] (25 µL) in 5% HCl (Hydrochloric acid, Sigma-Aldrich) (25 µL) and 2% sulphanilamide (Sigma-Aldrich) (25 µL), which were added to 50 µL of deproteinized samples in duplicate and kept in a water bath at 37 °C for 15 minutes. The optical densities were measured at 540 nm using a microplate reader (Epoch 2, BioTek). After that, nitrate was quantitated by reducing it to nitrite via the addition of vanadium (III) chloride (Sigma-Aldrich) (50 µL) to each sample, incubating for 15 min at 37 °C, and rereading absorbances at 540 nm. Total nitrite/nitrate (NO_2^-/NO_3^-) concentrations were calculated as µmol per liter of the plasma and urine using the standard curve generated from the serial concentrations of $NaNO_2$ (0-100 µmol/L) (Sodium nitrite, Sigma-Aldrich).

Quantitative RT-PCR analysis

Total RNA extraction, cDNA synthesis, and SYBR Green based-real-time PCR methods were performed to evaluate the expression levels of ACE2, iNOS, and apelin genes in rat kidney and heart samples. For RNA extraction, 150 mg of each kidney and heart tissue sample in BIR, BIR+RIPreC, and sham groups was homogenized by adding 800 µL Trizol (YTzol, Yekta Tajhiz Azma, Tehran, Iran) in sterile falcon tubes on water and ice (4 °C). Homogenized solutions were centrifuged for 5 minutes at 4000 rpm, and their supernatants were transferred to an RNase-free 2 mL microtube. In the next step, 400 µL chloroform (Sigma-Aldrich) was added, and the solution was kept at 4 °C for 5 minutes and centrifuged for 20 minutes at 13500 rpm and 4 °C to form aqueous and organic phases. The aqueous phase was removed to a new RNase-free microtube, 800 µL ethanol 100% was added, and it was kept overnight at -20 °C. The next day, this solution was centrifuged for 20 minutes at 13500 rpm and 4 °C. The supernatant was removed, 800 µL ethanol 75% was added, and the mixture was centrifuged for 8 minutes at 8000 rpm and 4 °C. Then, the supernatants were removed, and microtubes were dried under the hood station for 20 minutes. Total RNA sediment was solved in 50 µL diethylpyrocarbonate (DEPC)-treated water (Sinaclon, Karaj, Iran) and put on a dry block at 55-60 °C for 10 minutes to open the secondary structure of RNA. The purity and concentration of RNA were measured using NanoDrop™ (Thermo Scientific™, Waltham, Massachusetts, United States) at 260/280 nm, and cDNA synthesis was done for the rat kidney and heart samples using AddScript cDNA Synthesis kit, based on the manufacturer's instructions (AddScript cDNA Synthesis, AddBio Inc., Yuseong-gu, Daejeon, Korea).

ACE2, iNOS, and apelin expression levels in the kidney and heart tissue samples in all three groups were determined using an in-house SYBR green Real-time PCR protocol by Step One Real-Time Instrument (ABI, StepOnePlus, Foster City, United States). The

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was considered as an internal control to eliminate test errors.

The real-time PCR mix was composed of PCR master mix (Hot-start Taq DNA Polymerase, SYBR Green I dye, dNTPs mixture, protein stabilizers, and enhancers) (Add SYBR Master, AddBio Inc.), forward and reverse primers (10 pmol), and template cDNA. The real-time conditions of the studied transcripts were 95 °C/10 min, 40 cycles at 95 °C/15 s, 58°C/20 s, and 72 °C/30 s. Melting curves of the target and internal control genes were analyzed to confirm the specificity of PCR reactions. All real-time tests were repeated three times. The reverse and forward primer sequences for transcript amplification are shown in Table 1.

Kidney and heart histological evaluation

Renal and heart tissue samples were fixed in 10% formalin solution (Formaldehyde 37%, Merck Chemicals GmbH, Darmstadt, Germany). Slides of the samples were prepared at 5 µm thickness and stained with the routine hematoxylin and eosin (H&E) method.

In a blinded fashion, an expert pathologist examined each section in at least ten randomly selected non-overlapping fields under the light microscope. The renal histopathology was quantified for the degree of Bowman's space enlargement, brush border loss in proximal tubules, cast formation, and tubular necrosis. The myocardium structural disturbance was assessed based on the score of interstitial edema, minimum congestion, and leucocyte infiltration. The level of each manifestation was graded according to the changes involved, scoring 0 with no changes, 1 with less than 20%, 2 with 20–40%, 3 with 40–60%, 4 with 60–80%, and 5 with greater than 80%. The sum of all numerical scores in each group was reported as the total histopathological score.[7,28,29]

Statistical analysis

All data are expressed as mean ± SEM. Multiple comparisons between groups were performed using one-way ANOVA, followed by Dunnett's and Tukey's post hoc tests. Differences were considered statistically significant at $P<0.05$. Statistical analyses were performed using GraphPad Prism software package version 9. (GraphPad Software Inc. La Jolla, California, USA).

Results

Renal and cardiac injury biomarkers

As shown in Table 2, renal BIR caused a significant increase in the plasma levels of Cr and BUN compared to values obtained from the plasma of sham-operated animals. Cardiac injury markers, including LDH and troponin I were significantly higher in the BIR group than the sham group. The renal and cardiac functions improved when cyclic limb remote ischemia started along with the renal ischemia. Therefore, the plasma levels of Cr and LDH significantly increased, and BUN and troponin I slightly declined in the RIPerC group compared with the BIR group.

Blood pressure and electrocardiogram data

As determined in Table 3, mean blood pressure (MBP) significantly decreased in the BIR group compared with the sham group. The heart rate increased, but not

Table 1. Primer sequences for transcript amplification

Gene name	Gene ID	Sequence (5'→3')	Product length (bp)
GAPDH	24383	F: AGTGCCAGCCTC GTCTCATA	91
		R: GAGAAGGCAGCCCTGGTAAC	
ACE2	302668	F: GTGTCGTGATGGGAACGGTA	124
		R: TTGCCAATGTCCATGGAGTCA	
iNOS	24599	F: GGGAGAGTGAGCTGGTGTTT	108
		R: TGCAGTCCCGAGCATCAAAT	
Apelin	58812	F: CTCTGGCTCTCCTTGACTGC	109
		R: TCGAAGTTCTGGGCTTCACC	

Table 2. Effect of remote ischemic per-conditioning on renal and cardiac injury biomarkers

Variables		Experimental groups		
		Sham	BIR	BIR+RIPerC
Renal injury markers	Cr level (mg/dL)	0.49 ± 0.02	3.91 ± 0.42****	2.19 ± 0.28$^{***, \#\#\#}$
	BUN level (mg/dL)	16.63 ± 1.54	120.70 ± 5.15****	109.30 ± 7.58***
Cardiac injury markers	LDH (U/L)	404.80 ± 120.60	1816 ± 334.80**	1187 ± 114.40$^{\#}$
	Troponin I (ng/mL)	0.18 ± 0.07	0.58 ± 0.21**	0.23 ± 0.06

Data are expressed as mean ± SEM, n=10.
$^{**}P<0.01$, $^{***}P<0.0001$ and $^{****}P<0.0001$ vs. the sham group. $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ vs. the BIR group.
Cr, creatinine; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; BIR, bilateral ischemic reperfusion; RIPerC, remote ischemic per-conditioning.

Table 3. Effect of remote ischemic per-conditioning on the mean blood pressure, heart rate, intervals, and voltages of electrocardiogram waves

Variables	Experimental groups		
	Sham	BIR	BIR+RIPerC
MBP (mm Hg)	104.50 ± 8.40	88.55 ± 3.70**	102.90 ± 4.15#
HR (BPM)	215.10 ± 7.75	221.30 ± 9.85	232.50 ± 9.01
P-R Interval (ms)	53.43 ± 1.71	52.75 ± 1.55	55.83 ± 1.06
P Duration (ms)	23.53 ± 1.08	14.80 ± 0.78***	19.42 ± 1.05*,#
QRS Interval (ms)	15.26 ± 0.87	20.27 ± 1.02**	19.10 ± 0.52*
QTc Interval (ms)	41.18 ± 2.22	62.50 ± 2.44***	57.26 ± 3.65**
J-T Interval (ms)	22.40 ± 2.40	40.31 ± 2.90**	42.83 ± 2.70***
P Amplitude (mV)	1.62 ± 0.25	1.40 ± 0.25	1.70 ± 0.50
Q Amplitude (mV)	-4.25 ± 0.72	-3.38 ± 0.90	-2.70 ± 0.30
R Amplitude (mV)	4.46 ± 0.50	4.67 ± 1.13	4.22 ± 0.51
S Amplitude (mV)	-3.20 ± 0.90	-3.40 ± 0.96	-2.67 ± 0.22
ST Height (mV)	-2.84 ± 0.80	-3.37 ± 0.80	-2.33 ± 0.40
T Amplitude (mV)	4.07 ± 1.27	2.24 ± 0.90	2.10 ± 0.58

Data are expressed as mean ± SEM, n=10.
*P<0.05, **P<0.01, and ***P<0.001 vs. the sham group. #P<0.05 vs. the BIR group.
MBP, mean blood pressure; HR, heart rate; BPM, beat per minute; BIR, bilateral ischemia-reperfusion; RIPerC, remote ischemic per-conditioning.

significantly, in this group. ECG long lead II was recorded to evaluate whether the renal BIR induced functional disturbance in myocardia. ECG analysis data showed that P duration significantly decreased, whereas QRS, QTc, and J-T intervals significantly increased in the BIR group in comparison with the basic intervals in the sham group. Remote ischemic per-conditioning slightly modulated the transmission of the cardiac impulse. P duration was significantly higher in the BIR+RIPerC compared with the BIR group. Moreover, QRS and QTc intervals slightly declined in the BIR+RIPerC group; however, there were significant differences with the basis durations in the sham group. The P-R interval did not change in the three studied groups (Table 3).

Bilateral renal ischemia (60 minutes) with 24 hours reperfusion led to a slight decline in P and T amplitude as well as ST depression in the BIR group compared to the sham group; however, the difference was not significant. RIPerC restored the P and T amplitude and ST depression to the basal voltage in the BIR+RIPerC group. No significant differences in Q, R, and S voltages were observed among the three experimental groups (Table 3).

ACE activity in the plasma and tissue

The activity of the ACE was measured in three types of samples (plasma, heart, and kidney) 24 hours after reperfusion. Our data showed that bilateral renal ischemia (60 minutes) stimulated the activity of ACE. Therefore, the activity of ACE in the kidney, as well as plasma and heart tissues, was significantly elevated in the BIR group compared to that in the sham group (in all three P<0.0001). Cyclic remote ischemia at the time of ischemia prevented the increase in the ACE activity seen after renal ischemia. For this reason, there was a significant difference in the ACE activity between the BIR+RIPerC and BIR groups in the plasma and heart (P<0.0001) as well as in the kidney tissue (P<0.001). However, the kidney ACE activity in the BIR+RIPerC group was higher than the sham group (P<0.01) (Fig. 1).

NO metabolites (nitrite and nitrate) in the renal and cardiac tissues

We measured the concentration of NO metabolites (nitrite and nitrate) in the kidney and heart tissues. Our data confirmed that NO production was induced after renal ischemia in the kidney as well as heart tissue. Thus, tissue NO_2^- (nitrite) levels were elevated in the BIR group compared with the sham group (P<0.05). In addition, the kidney and heart NO_3^- (nitrate) levels slightly increased after renal ischemia in the BIR group. While nitrite and nitrate content decreased in the BIR+RIPerC group, there was no significant difference between the mean values of NO_2^- and NO_3^- levels in the BIR and BIR+RIPerC groups (Table 4).

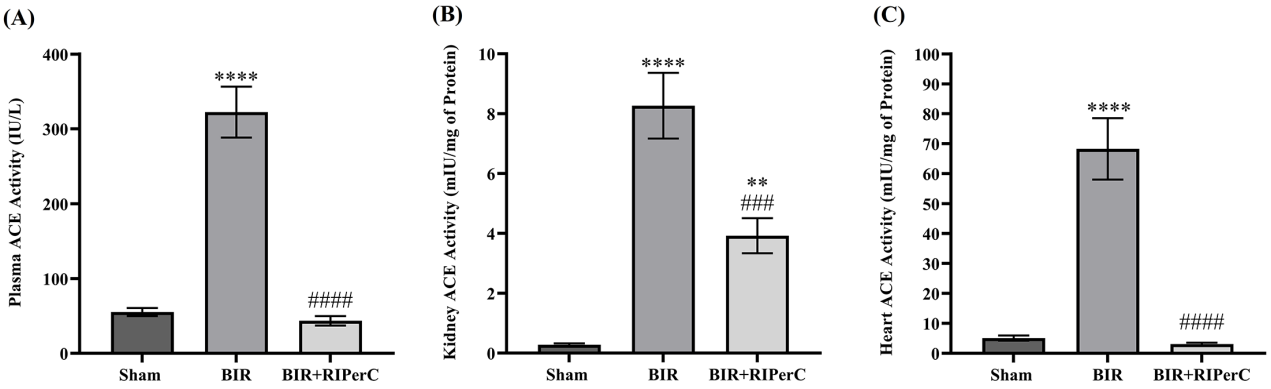

Fig. 1. Effect of limb remote ischemic per-conditioning on the activity of the angiotensin-converting enzyme (ACE) in the plasma (A), kidney tissue (B), and heart tissue (C). Data are expressed as mean ± SEM, n=10. **P<0.01 and ****P<0.0001 vs. the sham group. ###P<0.001 and ####P<0.0001 vs. the BIR group.

Table 4. Effect of remote ischemic per-conditioning on the concentration of NO metabolites (nitrite and nitrate) in the kidney and heart tissues

Variables		Experimental groups		
		Sham	BIR	BIR+RIPerC
Kidney	NO_2^- level (µmol/L)	53.01 ± 6.79	76.43 ± 7.52*	72.96 ± 3.44
	NO_3^- level (µmol/L)	2.04 ± 0.06	2.11 ± 0.11	1.91 ± 0.03
Heart	NO_2^- level (µmol/L)	43.14 ± 7.14	65.60 ± 4.61*	58.04 ± 3.99
	NO_3^- level (µmol/L)	1.77 ± 0.08	1.90 ± 0.12	1.78 ± 0.02

Data are expressed as mean ± SEM, n=10.
*P<0.05 vs. the sham group.

NO metabolites (nitrite and nitrate) in the biological fluids

We also investigated the level of nitrite and nitrate in the biological fluids (plasma and urine) samples in the experimental groups as an index of NO synthesis. There was a significant increase in the plasma and urine nitrite and nitrate concentration in the BIR compared with the sham group (P<0.01 and P<0.05, respectively). Increased plasma and urine nitrite and nitrate levels induced by renal BIR were significantly decreased in BIR+RIPerC in comparison with the BIR group (P<0.0001 and P<0.001, respectively) (Fig. 2).

The mRNA levels of apelin, ACE2, and iNOS in the renal and heart tissues

After 24 hours of reperfusion, the apelin mRNA expression was downregulated in the renal tissue (Fig. 3A), not the cardiac tissue (Fig. 3D) of the BIR group. However, the expression levels of ACE2 and iNOS in renal and myocardium tissues were significantly higher in the BIR groups than in the sham group (Fig. 3B-C and Fig. 3E-F). Remote ischemic per-conditioning modulated the expression of these genes. The apelin expression was upregulated, and iNOS and ACE2 expression were downregulated in BIR+RIPerC compared to the BIR group in both studied tissues (Fig. 3A-F).

Renal and cardiac histopathological findings

Renal glomerular, tubular, and vascular damages in the experimental groups are shown in detail in Fig. 4. The normative appearance was found in the sham group (Fig. 4A and 4D) in the cortex and medulla area. Compared with those in the sham group, the rats in the BIR group showed significant renal histopathologic changes,

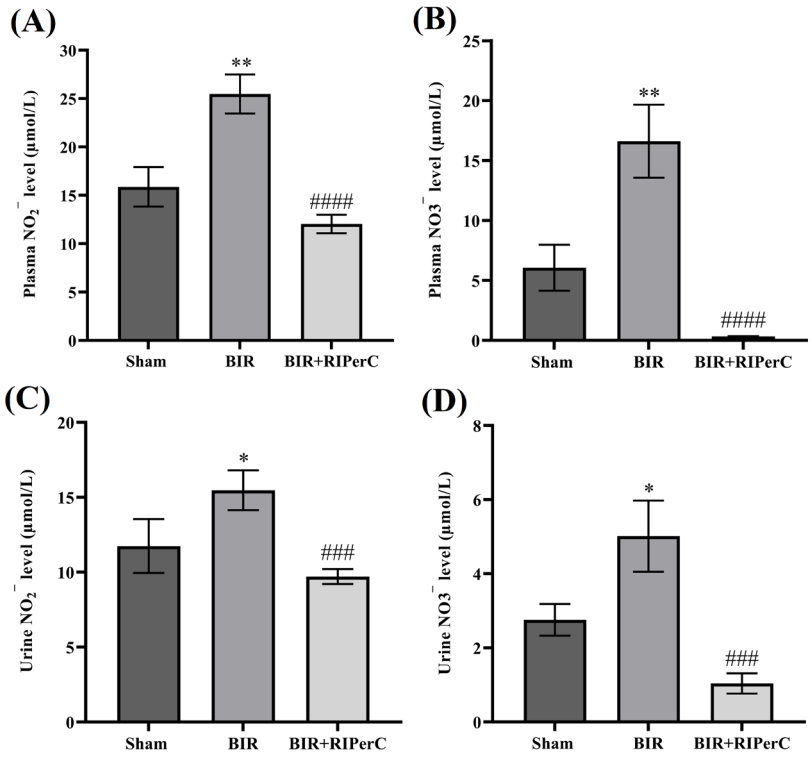

Fig. 2. Effect of remote ischemic per-conditioning on NO metabolites (nitrite and nitrate) in the biological fluids (NO_2^- and NO_3^- levels in the plasma (A and B) and urine (C and D) samples, respectively). Data are expressed as mean ± SEM, n=10. *P<0.05 and **P<0.01 vs. the sham group. ###P<0.001 and ####P<0.0001 vs. the BIR group. BIR, bilateral ischemic reperfusion; RIPerC, remote ischemic per-conditioning.

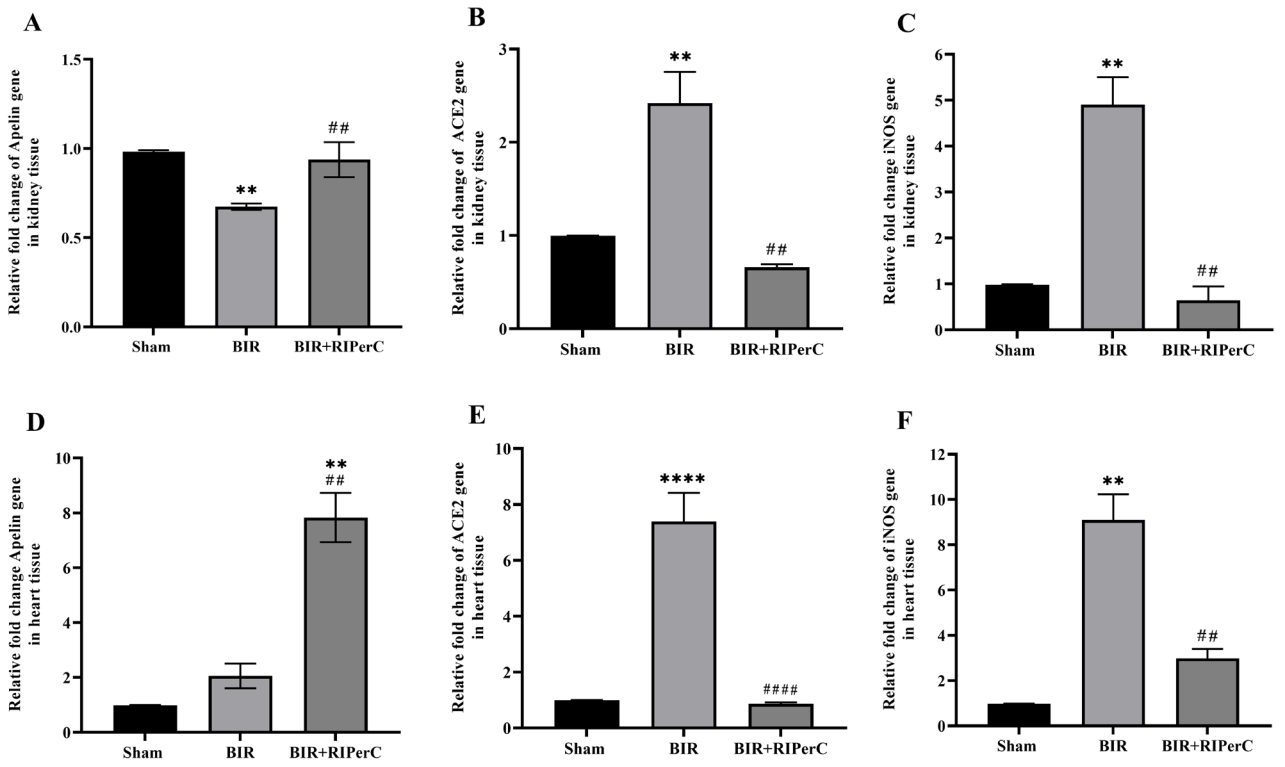

Fig. 3. Effect of remote ischemic per-conditioning on the apelin (A and D), ACE2 (B and E), and iNOS (C and F) mRNA expression in the renal and heart tissues. Data are expressed as mean ± SEM, n=10. $^{**}P<0.01$ and $^{****}P<0.0001$ vs. the sham group. $^{\#\#}P<0.01$ and $^{\#\#\#\#}P<0.0001$ vs. the BIR group. BIR, bilateral ischemic reperfusion; RIPerC, remote ischemic per-conditioning.

including bowman space enlargement, epithelial tubular cells injury, acute tubular necrosis, intratubular cast, and vascular congestion (Fig. 4B and 4E). In the BIR+RIPerC group, minimal structural changes in the glomeruli and tubules were observed (Fig. 4C and 4F). Quantifiable results showed that the total histopathological score significantly increased in the BIR group and modulated in the BIR+RIPerC group (Fig. 4G).

Bilateral renal ischemic reperfusion-induced slight structural damage in the heart tissue. The appearance of the sham group did not show alterations. Myocardial tissue destruction was characterized by interstitial edema, minimum congestion, and leucocyte infiltration in the BIR group. The combination of renal ischemia with cyclic remote ischemia (femoral artery ischemia) attenuated the severity of lesions in the BIR+RIPerC group. The total histopathological score decreased in the BIR+RIPerC group (Fig. 5A-D).

Discussion

Renal ischemia/reperfusion-induced AKI is an outcome of various clinical situations, including transplantation, partial nephrectomy, sepsis, hydronephrosis, or elective urological surgery. Recent studies have demonstrated that cardiac failure is the most common complication after clinical and experimental AKI induced by renal ischemia/reperfusion.[10,30]

The obtained results in this study demonstrated that renal ischemia induced cardiac functional disturbance and structural damage. In addition, we detected significant elevation in ACE activity, No metabolites, and mRNA levels of iNOS and ACE2 in the post-renal I/R (ischemic/reperfusion) heart tissue.

Renal I/R injury caused histological and functional alterations in renal and heart tissues.[30-32] We also found that tissue damages were associated with high plasma levels of renal and cardiac injury biomarkers, such as BUN, Cr, LDH, and troponin I.

Remote ischemic conditioning (RIC) is an adaptive phenomenon that causes tissue resistance to long-term ischemia or ischemia-reperfusion after one or more brief conditioning ischemia-reperfusion cycles.[33,34] The phenomenon of RIC was first reported by Murry et al.[35] Some researchers separately showed renal and cardioprotective effects of remote ischemic per-conditioning.[36,37] To the best of our knowledge, no study has reported the mechanism involved in the protective effect of RIPerC on acute heart injury due to renal I/R injury. For the first time, we investigated the protective effects of this procedure on cardiac injury induced by renal I/R injury through interactive effects of apelin, RAS axis, and iNOS. In the first step of this research, we found that renal as well as heart dysfunctions improved when renal ischemia-reperfusion was done along with remote ischemic per-conditioning. The post-renal I/R myocardium injury improved with RIPerC. According to our previous study,

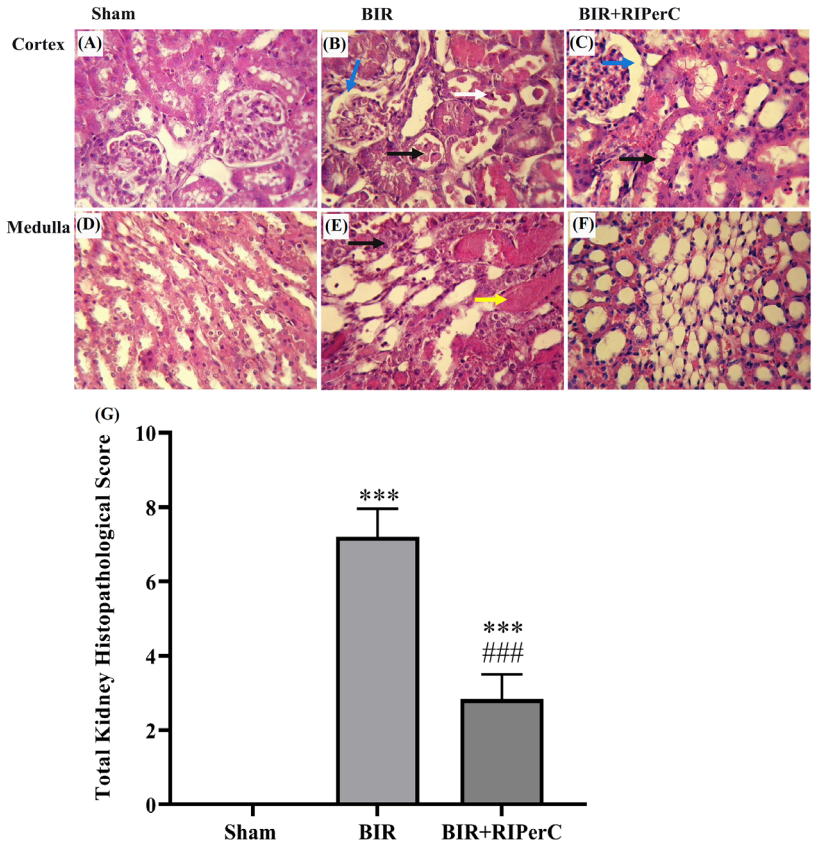

Fig. 4. Histopathological finding of the renal cortex and medulla at the end of 24 h reperfusion: sham group (A and D) showed normal appearance. Obvious tissue injuries were observed in the BIR group (B and E); a higher degree of Bowman space enlargement (blue arrow), loss of brush border in the proximal tubules (black arrow), cast formation (yellow arrow), and tubular necrosis (white arrow). Remote ischemic per-conditioning improved structural changes in the BIR+RIPerC group (C and F). The level of each manifestation was graded according to the changes involved, scoring 0 with no changes, 1 with less than 20%, 2 with 20–40%, 3 with 40–60%, 4 with 60–80%, and 5 with greater than 80%. The sum of all numerical scores in each group was stated as the total histopathological score (G). H & E staining (magnification ×400) and data are expressed as mean ± SEM, $^{***}P<0.01$ vs. the sham group and $^{###}P<0.001$ vs. the BIR group. BIR, bilateral ischemic reperfusion; RIPerC, remote ischemic per-conditioning

histopathological findings demonstrated that remote ischemic per-conditioning significantly decreased epithelial tubular cell injury, tubular necrosis, intratubular cast, and vascular congestion.[38]

Moreover, other cardiac functional data indicated that RIPerC causes amelioration of ECG parameters, mean blood pressure, and heart rate. Prolonged QTc and QRS intervals in the BIR groups are similar to those of other studies on the cardiac effect of renal I/R injury.[39,40] These results and the rise in troponin I and LDH activity confirmed myocardial injury after renal I/R injury.

However, the mechanisms involved in the cardiac injury induced by renal I/R are not completely understood. In the second part of the study, we set out to investigate the interactive effect of RAS, iNOS, and apelin in our experimental groups. Our result showed that apelin plays a critical role in post-renal I/R cardiac dysfunction.

Various studies have indicated that endogenous apelin/APJ receptor has potential therapeutic effects in kidney and cardiovascular diseases.[17,41,42] Wang et al showed that apelin mRNA downregulated at 1-day post-myocardial infarction (MI) in humans and murine. In addition, apelin-knockout mice were susceptible to MI after cardiac ischemia-reperfusion.[43] Moreover, Gholampour et al showed that apelin expression increased after renal I/R injury.[44] However, our result indicated that the expression level of the apelin gene decreased in the kidney, but not the heart, after renal I/R injury, whereas apelin mRNA upregulated in the heart and kidney of the BIR+RIPerC rats.

During renal ischemia and re-oxygenation of the tissue, the parenchymal cells, including tubular epithelial cells and vascular endothelial cells, generate free radicals, which are reactive nitrogen species (RNS) or reactive oxygen species (ROS). ROS/RNS stimulates inducible isoforms of NOS, which causes about 1000-fold higher NO formation than eNOS.[11,45]

Our results were in line with those of Meng et al study that indicated mRNA levels of iNOS increased after renal I/R in the kidney sample.[46] In addition, nitrate and nitrite concentration as an index of NO production increased in biological fluids (blood and urine). Previous studies have confirmed systemic redox imbalance and systemic inflammation after renal I/R.[10,47,48] Therefore, in

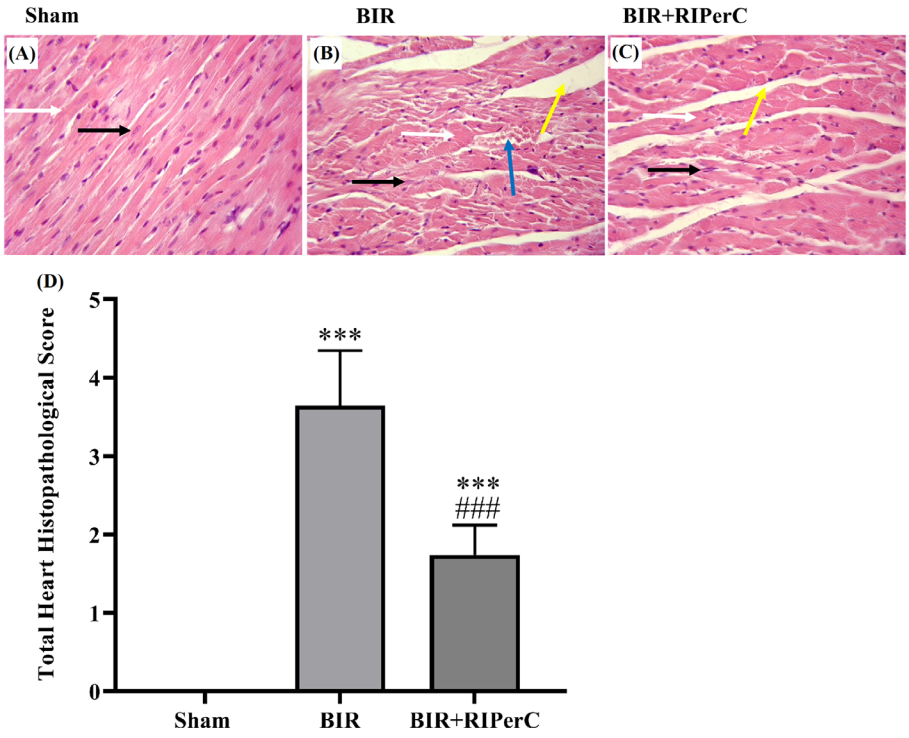

Fig. 5. Histopathological finding of the heart tissue at the end of 24 h reperfusion: sham group (A) showed a normal appearance. Obvious tissue changes were detected in the BIR group (B) with a higher degree of interstitial edema and congestion. Remote ischemic per-conditioning improved the structural changes in the BIR+RIPerC group (C). Muscle fiber (white arrow), central nucleus (black arrow), congestion (blue arrow), and interstitial edema (yellow arrow). The level of each manifestation was graded according to the changes involved, scoring 0 with no changes, 1 with less than 20%, 2 with 20–40%, 3 with 40–60%, 4 with 60–80%, and 5 with greater than 80%. The sum of all numerical scores in each group was stated as the total histopathological score (D). H & E staining (magnification ×400) and data are expressed as mean ± SEM, $^{***}P<0.01$ vs. the sham group and $^{###}P<0.001$ vs. the BIR group. BIR, bilateral ischemic reperfusion; RIPerC, remote ischemic per-conditioning

the current study, upregulation of cardiac iNOS mRNA may be related to response to these systemic conditions. Overexpression of cardiac iNOS was accompanied by increased nitrate and nitrite levels in the post-renal I/R myocardium. In two separate studies, Güvenç et al and Feng et al demonstrated the deleterious effect of NO derived from iNOS.[49,50] On the other hand, in accordance with other studies, some cardiovascular protective effects of the apelin peptide depend on NO derived from eNOS.[19,51] An et al reported that apelin overexpression increased the eNOS levels through diminishing iNOS levels during ischemia-reperfusion injury in diabetic myocardium.[52] In the current study, we found decreased apelin expression along with increased iNOS expression in the tissue samples of the BIR group.

Interestingly, our result demonstrated that iNOS expression following NO production decreased when renal ischemic reperfusion was performed along with remote ischemic per-conditioning. Accordingly, nitrate and nitrite concentration declined in the kidney and heart tissues and biological fluids.

In the third step, we measured different components of the cardiac and renal RAS in the experimental groups to evaluate the association between the RAS axis and apelin activity.

Renal I/R seems to change the balance of the RAS axis.[3,53] This is supported by our data which showed that ACE activity markedly increased in the plasma as well as cardiac and renal tissues 24 h after reperfusion. Efrati et al demonstrated that captopril, as an ACE inhibitor, declined angiotensin-II production following the development of renal dysfunction at the early stage of reperfusion, differentially inhibited inflammation, significantly decreased intrarenal NO, and reduced histopathologic signs of renal damage after renal I/R.[13] Interestingly, in our work, ACE2 mRNA was upregulated in both tissue samples in the BIR group. Whereas da Silveira Kátia et al detected that gene expression of ACE2 remained unchanged in the renal I/R2h group, it was significantly downregulated in the I/R4h group.[54] The increased level of ACE2 mRNA in the heart and renal tissue may be a compensatory response to the local and systemic increase of the ACE activity in ischemic conditions, even though the elevation of the mRNA level of ACE2 could not suppress the deleterious effect of ACE activity in the ischemic condition.

Clear evidence exists of antagonism between the apelin system and the RAS. Previous studies have confirmed that apelin is an endogenous and cardioprotective peptide that exhibits beneficial effects through the modulation of RAS axis components in the pathology of cardiovascular

Research Highlights

What is the current knowledge?

√ Remote ischemic conditioning upregulates endogenous protective pathways in response to ischemia-reperfusion injury.

√ The physiological and pathophysiological interaction between the kidney and the cardiovascular system provides essential indicators for maintaining homeostasis.

What is new here?

√ Limb remote ischemic per conditioning improves the myocardium dysfunction induced by renal ischemia-reperfusion injury through the interaction of the apelin with the RAS/iNOS pathway.

diseases.[55] Apelin depletion progressed the myocardium dysfunction and structural remodeling through angiotensin II pathways.[56] Our data clearly indicated that remote ischemic per-conditioning suppressed the cardiac ACE activity and ACE2 expression.

Conclusion

Remote ischemic per-conditioning is a potential phenomenon to promote endogenous protective pathways against renal I/R injury. The findings of the current study revealed that renal I/R induced kidney and heart structural and functional disturbance by a significant increase in injury biomarkers. Histological damages in the renal and heart tissues were in line with the increased ACE activity and NO metabolites in the tissues and biological samples. On the other hand, the level of apelin mRNA was downregulated, but iNOS and ACE2 genes were upregulated in the renal and heart tissues. In total, our results indicated that RIPerC protects the heart against renal I/R injury, probably through the interaction of the apelin with the RAS/iNOS pathway.

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Competing interests

The authors declare no conflict of interest.

Ethical Statement

The experimental protocols were approved by the local ethics committee of Shiraz University of Medical Sciences (Approval ID: IR.SUMS.REC.1399.894).

Data Availability Statement

Data reported in this manuscript are available upon reasonable request from the corresponding author.

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