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Utility of Combined Ischemic Pre- and Post-Conditioning to Protect the Human Myocardium

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Abstract

Objective: Ischemic pre-conditioning (IPreC) and post-conditioning (IPostC) protect the human myocardium against ischemia/reoxygenation (I/R) injury. However, the two interventions may induce variable degrees of protection, suggesting different mechanisms of action. This study assessed whether IPreC and IPostC confer greater protection when used in combination rather than individually.

Methods: The right atrial appendages from 50 patients were subjected to 90 min of ischemia and 120 min of reoxygenation according to different protocols: IPostC (1 cycle of 120 and 180 sec of I/R) and IPreC (1 cycle of 5 min ischemia/5 min reoxygenation), alone and in combination. Lactate dehydrogenase (LDH) release was measured as an index of tissue injury and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction as an index of cell viability.

Results: The results showed that in one half of muscles the combined use of IPreC and IPostC reduced injury compared to either intervention alone whereas in the remaining half the combined approach had no greater effect. Nonetheless, the addition of IPreC to IPostC increased the number of protected samples by almost 20% compared to IPostC alone.

Conclusion: The results demonstrate the lack of a uniform response to IPreC and IPostC and that the combined use of the two treatments improves protection although does not abolish the further myocardial injury that occurs in some instances in response to IPostC.

Keywords: Ischemia or reperfusion; Ischemic pre-conditioning; Ischemic post-conditioning; Human myocardium

Introduction

Ischemic injury due to coronary artery disease is the most frequent cause of death and disability in developed countries [1]. However, the prevention and effective limitation of myocardial damage remains an unmet clinical need. Ischemic pre-conditioning (IPreC) and ischemic post-conditioning (IPostC) are effective in different experimental animal models [2] but the results in humans are controversial [3-7]. We previously observed that the myocardium of approximately 2/3 of the patients with underlying heart diseases was protected by IPreC and approximately 1/3 by IPostC [8]. An explanation for the discrepancy between humans and the animal models may be that IPreC and IPostC protocols are well-adapted to animal experimental conditions but may not necessarily be maximally effective in humans. Using *in vitro* isolated human myocardium, we previously demonstrated that among the IPreC protocols tested the most effective one consisted of a cycle of 5 min of ischemia followed by 5 min of reoxygenation [9], and the most effective IPostC protocol was a single cycle of 2 min of ischemia [8]. The association between heart diseases and cardiovascular risk factors may also influence ischemic conditioning of the myocardium. Thus, we recently showed a lower protective response to IPreC of myocardium from patients with mitral valve disease than from patients with other heart conditions, and that myocardial protection by IPreC was greater in females than in males [10].

The mechanisms of IPreC- and IPostC-mediated protection have yet to be fully elucidated but they probably share one or more molecular and cellular pathways [11-14]. However, whether the combined application of IPreC and IPostC affords a greater degree of protection than either treatment alone or whether only a subset of patients will benefit from both treatments is unknown. Previous studies using different experimental approaches produced contradictory results [15-17]. Therefore, in the present study, we chose a well-characterized model of ischemia/reoxygenation and well defined protocols for IPreC [9] and IPostC [8] to investigate the protective potential of the combined use of the two interventions in samples of human myocardium.

Methods

Population

The study was approved by the Ethics Committee of Clinical Investigation at Vall d'Hebron University Hospital (ID-RTF065), and informed consent was obtained from each participating patient.

The right atrial appendage was obtained from patients undergoing elective cardiac surgery prior to cannulation of the heart and the

establishment of cardiopulmonary bypass. The anesthetic protocol was identical in all donor patients. Demographic data, the presence of vascular risk factors, previous morbidities, and medications were recorded. Fifty patients were sequentially enrolled without any exclusion criteria.

Study design

The right atrial appendages were collected in Krebs Henseleit Hepes buffer (KHH) [(mM): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), $MgCl_2$ (1.2), $KH2PO_4$ (1.0), $CaCl_2$ (1.25), HEPES (20), pH 7.4] at 4°C and processed as previously described [9]. Surgical skin graft blades (Swann-Morton, Sheffield, UK) were used to prepare tissue slices with a thickness of 300–500 μm. The muscle slices (weight 30–50 mg each) were then transferred to Erlenmeyer flasks (Trallero and Schlee, Barcelona, Spain) containing 10 ml of oxygenated KHH (pH 7.4) at 37°C supplemented with 10 mM d-glucose (Sigma, St. Louis, MO). The flasks were then placed into a shaking water bath at 37°C. Simulated ischemia was induced for 90 min by bubbling the KHH with 95% N_2-5% CO_2 (pH 6.80–7.00) and replacing the d-glucose with 2-deoxy-d-glucose (Sigma, St. Louis, MO). The muscles were then reoxygenated for another 120 min. Some muscles were aerobically incubated at 37°C for the entire experiment whereas others were subjected to IPreC, induced by 5 min of ischemia followed by 5 min of reoxygenation prior to the 90 min of ischemia, a protocol shown to elicit optimal protection in this model [9]. Other muscles were subjected to IPostC, induced by 1 cycle of 120 and 180 sec of reperfusion and ischemia before the 120 min of reoxygenation; this protocol has also been previously shown by our laboratory to be the most effective under the tested conditions [8]. Muscles from each patient were allocated to the following groups (Figure 1): (i) aerobic control (AC); (ii) ischemia/reoxygenation alone (I/R alone); (iii) IPostC induced by 1 cycle of 120 and 180 sec of reperfusion and ischemia before the 120 min of reoxygenation; (iv) a combination of the IPostC and IPreC protocols; and (v) IPreC alone.

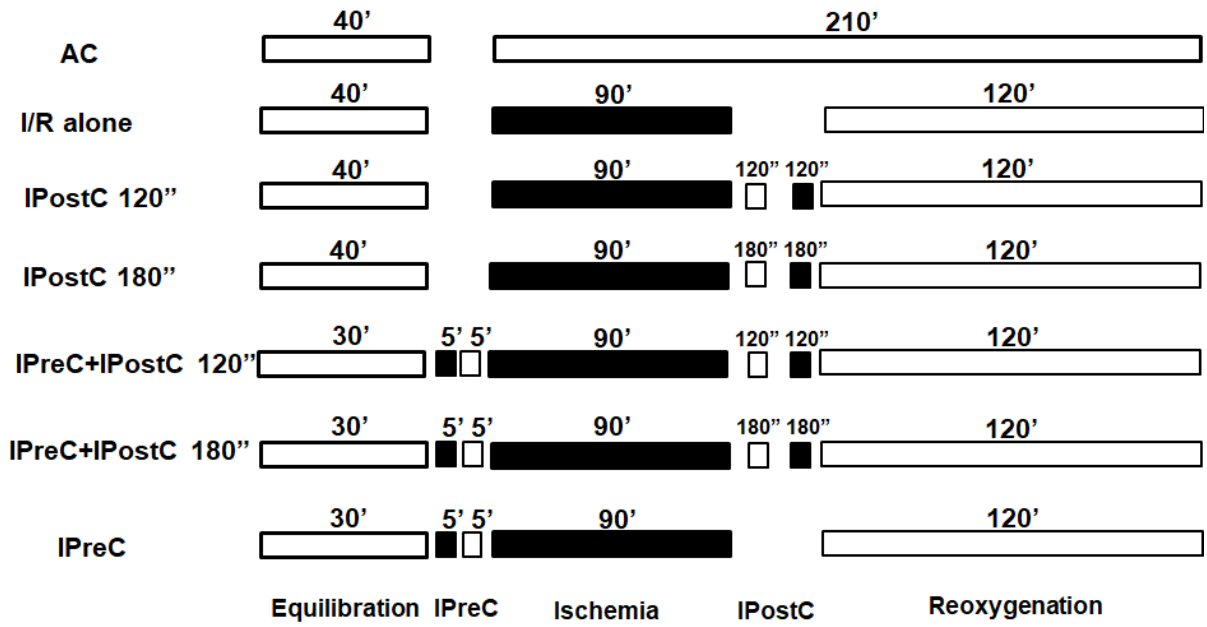

Figure 1: Experimental protocols. Muscle slices were equilibrated under aerobic conditions for 30-40 min at 37°C and then subjected to 90 min of ischemia followed by 120 min of reoxygenation (I/R alone group). Muscle slices from each patient were subjected to the following conditions, alone or in combination: ischemic post-conditioning (IPostC) induced by 120 sec of reoxygenation followed by 120 sec of ischemia and 180 sec of reoxygenation followed by 180 sec of ischemia, applied before the reoxygenation period; ischemic pre-conditioning (IPreC), induced by 5 min of ischemia followed by 5 min of reoxygenation and a combination of IPreC and IPostC. Some muscle slices were maintained under aerobic conditions (AC) during the entire experimental period.

Assessment of myocardial tissue injury and viability

Tissue injury was assessed at the end of the 120 min of reoxygenation by the leakage of lactate dehydrogenase (LDH), which converts pyruvate to lactate in the medium. The absorbance was measured at a 340 nm wavelength with a MultiSkan FC spectrometer (Thermo Fisher Scientific) and the results were expressed as arbitrary units (AU)/g wet tissue.

Tissue viability was assessed at the end of the 120 min of reoxygenation by the reduction of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO) to a blue formazan product. The newly formed formazan was measured at a wavelength of 550 nm using the same spectrometer as described above and the results were expressed as AU/g wet tissue.

Statistical analysis

The data were analyzed after subtraction of the aerobic control value and expressed as the mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) and the Kruskal-Wallis test were used to compare the means between all study groups and compared with I/R alone group. Linear regression and logistic binary regression were also used to compare the effect of co-morbid conditions and medical

treatments. A uni-variate analysis was performed to study the effect of the concomitant cardiac pathologies, the associated co-morbid conditions and the medical treatments received by the right atrial appendage. Statistical analyses were performed using SPSS 20 and GraphPad Prism 6. A $P<0.05$ was considered to indicate statistical significance.

Results

The demographic characteristics of the study patients, their associated clinical comorbid conditions, and the type of heart disease are listed in Table 1.

Variable	N = 50
Gender (M/F)	36/14
Age	64 ± 1.5
Obesity	16(32%)
Diabetes	14(28%)
Dyslipidemia	28(56%)
Hypertension	33(66%)
Coronary artery disease	21(42%)
Ascending aortic aneurysm	18(36%)
Interatrial communication	2(4%)
Left ventricular fraction ejection	
≥40%	47(96%)
≤40%	3(6%)
Atrial Fibrillation	
Paroxysmal	3(6%)
Permanent	11(22%)
Aortic valve disease	24(48%)
Mitral valve disease	7(14%)
Tricuspid valve disease	1(2%)

Table 1: Patient's demographic data.

Figures 2A and 2B show the mean LDH leakage and MTT reduction values for all groups of patients, respectively. Together, the data demonstrate that IPreC reduces myocardial damage (LDH leakage) and increases cell viability (MTT reduction) whereas IPostC increases damage and does not influence cell viability in the 120 sec and 180 sec protocols. When the IPreC and IPostC protocols were combined, the increased damage induced by IPostC was abolished but cell viability was reduced.

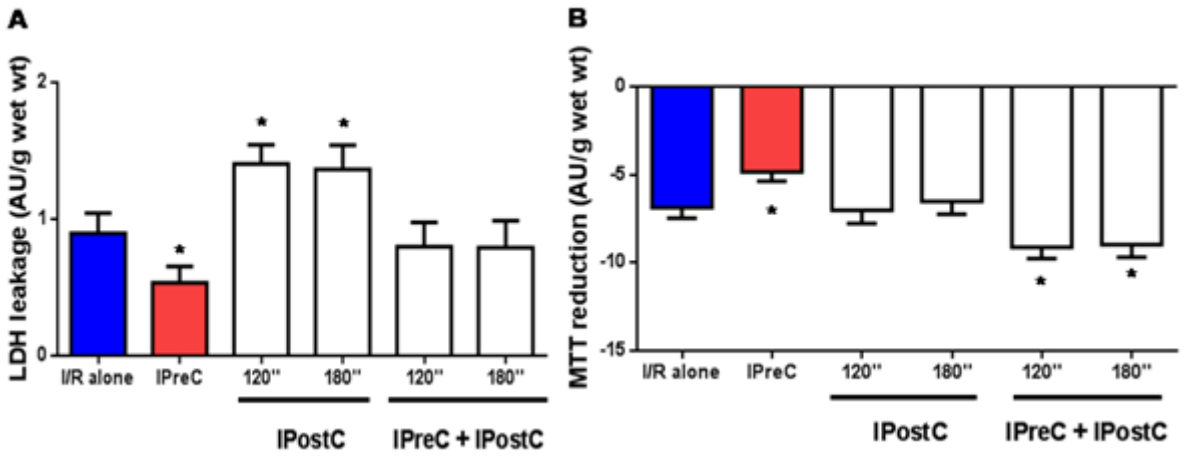

Figure 2: Effects of ischemic pre-conditioning (IPreC), ischemic post-conditioning (IPostC), and the combination of the two on lactate dehydrogenase (LDH) leakage (A) and MTT reduction (B) in human right atrial myocardium subjected to 90 min of ischemia followed by 120 min of reoxygenation (n=50); *$p<0.05$ *vs.* ischemia/reperfusion (I/R) alone.

Figure 3(A1) shows the results on LDH leakage of the different treated groups compared to the values obtained with I/R alone. It can be seen that 60% of the IPreC-treated samples exhibited LDH leakage values below (0.25 AU/g wet wt, $p<0.05$) those of the corresponding I/R alone group (0.8 AU/g wet wt) whereas this was the case in only 26%–30% of the IPostC samples (0.5 and 0.26 AU/g wet wt, in the 120 and 180 sec groups, respectively). Importantly, the combined use of IPreC and IPostC further reduced LDH leakage in 46%–50% of the samples and the levels were significantly lower (eg, lesser myocardial injury with values between -0.1 and 0.03 AU/g wet wt) than those obtained by applying either IPreC or IPostC alone.

Figure 3(B1) shows the MTT reduction values; IPreC afforded better protection in 60% of the cases (-3.6 AU/g wet wt), while the percentage was reduced between 44%–48% by the IPostC treatment (-3.91 and -3.11 AU/g wet wt in the 120 and 180 sec groups, respectively) and between 24%–50% with the combination of the two treatments (-4.4 and -4.5 AU/g wet wt, 120 and 180 sec groups, respectively). In samples in which LDH leakage (Figure 3 (A2)) and MTT reduction (Figure 3 (B2)) values were worse than their I/R alone

counterparts, the degree of damage was significantly greater with IPostC than with IPreC and the combined use of the two treatments did not change the mean values compared with those obtained with IPostC alone.

The study was not powered to identify a relationship of the use of IPreC and IPostC in combination with the concomitant cardiac pathologies, the associated co-morbid conditions and the medical treatments received and, accordingly, none of these factors were found to have an effect (data not shown).

Discussion

Using an *in vitro* model of ischemia/reoxygenation of the human right atrial appendage, the present study has shown that the combined use of IPreC and IPostC further reduces ischemic injury compared to either intervention alone in almost half of the cases, but it has no effect in the other half, as assessed by LDH leakage measurement. The addition of IPreC resulted in significant benefit based on LDH and MTT assays, increasing the number of protected samples by almost 20% compared to IPostC alone. However, this important finding was not apparent during the global examination of the results and only became evident after a more specific retrospective analysis (Figure 3). The observed variability in the LDH and MTT data may have been due to the differential effects of the two interventions on tissue injury and cell death. Thus, while IPreC yielded similar improvements in tissue injury and cell death, IPostC reduced cell death to a greater extent than tissue injury. These findings suggest that the protection conferred by IPreC and IPostC involves similar but also distinct pathways. Indeed, there is evidence in the literature that cellular pathways such as phosphatidyl inositol 3-kinase-Akt and extracellular signal-regulated kinase 1, nitric oxide, and mitochondrial KATP channels participate both in IPreC and IPostC [12,13,18], but also that the two interventions have different specific mechanisms that lead to protection, including activation of reperfusion-induced salvage kinase, protein kinase C, and the JAK–STAT (Janus kinase-signal transducer and activator of transcription) pathway [11,14,19].

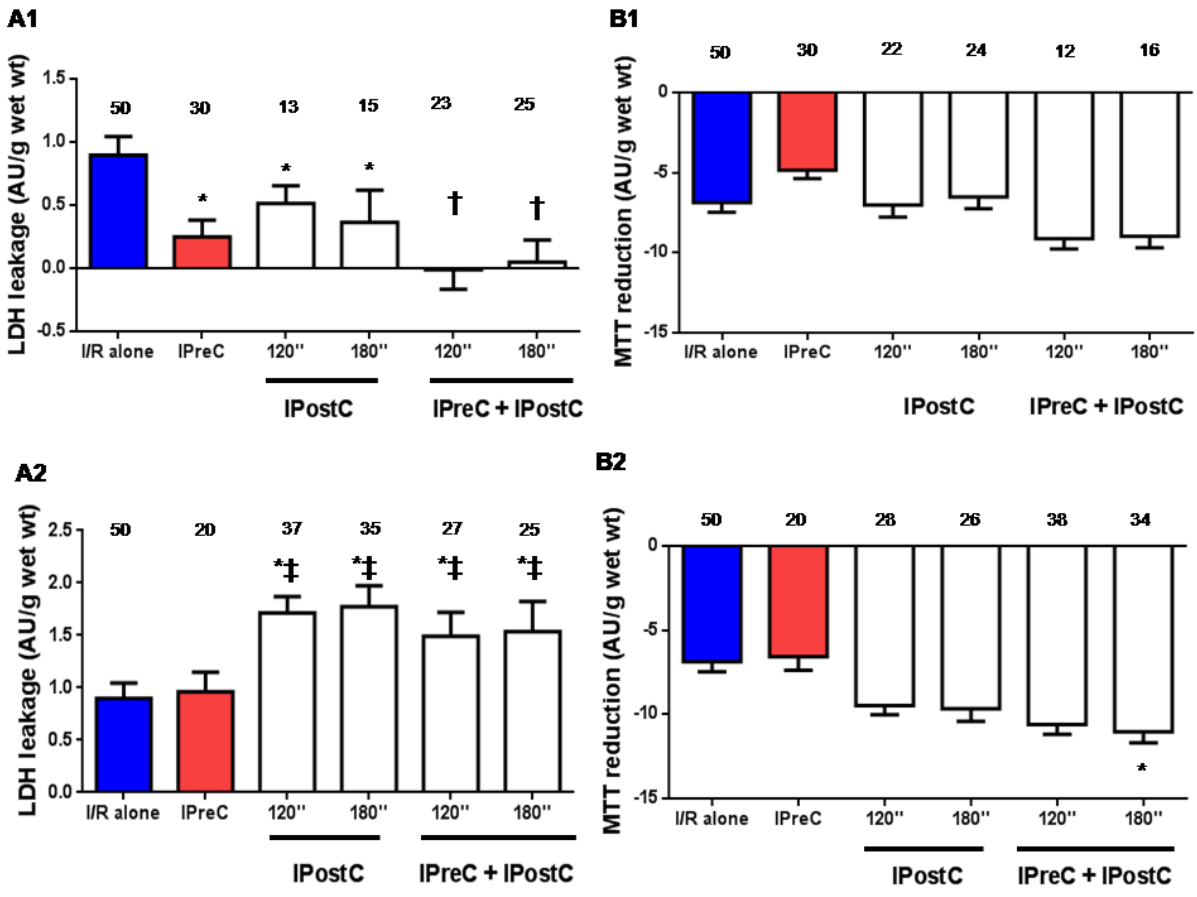

Figure 3: Separation of the LDH leakage (A1 and A2) and MTT reduction (B1 and B2) results for ischemic pre-conditioning (IPreC), ischemic post-conditioning (IPostC), and their combination according to whether the values were above or below the respective ischemia/reoxygenation (I/R) alone group; $^{*}p<0.05$ *vs.* I/R alone; $^{\dagger}p<0.05$ *vs.* IPostC or IPreC alone; $^{\ddagger}p > 0.05$ *vs.* IPreC alone.

If the incremental protection against myocardial injury seen with the combination of IPreC and IPostC may be interpreted as the utilization of different but complementary cellular mechanisms, it is speculated that the observed worsening in myocardial injury (LDH leakage) is caused by a defect in the cellular signal transduction mechanisms of protection and that, in fact, any additional ischemia at the start of the long period of ischemia or during reperfusion may aggravate tissue viability. Yet, although all the donor patients received

identical anesthetic protocol in which intravenous propofol was routinely administered and the atrial samples were obtained prior to the administration of cardioplegia and the induction of hypothermia, it cannot be fully discarded that the endogenous cellular transduction mechanisms could have been activated before collecting the right atrial samples. Indeed, the elucidation of these issues would require further studies including the investigation of the role played by specific elements of the cell signaling pathways.

Our results are in contrast with those from previous animal and human studies. Halkos et al. [20], in a study using dogs, and Manintveld et al. [21] and Tsang et al. [13] in their studies in rats, found that the combination of IPreC and IPostC did not have an additive effect. By contrast, other authors, in a rabbit study [18], showed that the combined use of the two interventions provided greater protection than either treatment alone. The different IPreC and IPostC protocols and animal species used in these studies might have contributed to the conflicting results.

Our findings of increased protection when IPreC and IPostC were used in combination in almost half of the cases using human myocardium in an *in vitro* model of ischemia/reoxygenation are supported by the results of the Lipsia Conditioning clinical trial [17], in which the combination of remote IPreC and IPostC in 696 patients with STEMI (ST-segment elevation myocardial infarction), scheduled for primary percutaneous coronary intervention, significantly increased myocardial salvage compared to either the control or IPostC alone. However, using muscular trabeculae from human right atrial appendages in an *in vitro* model of hypoxia, Roleder et al. [16] reported that the combined application of hypoxic pre-conditioning and post-conditioning abolishes the protection conferred by each intervention alone. Again, the variable results can probably be explained, at least in part, by differences in the type of clinical application and in the protocols used. Thus, there were notorious differences between the two *in vitro* studies (Rodeler et al. [16] and ours) both in terms of experimental models and protocols used: (i) simulated ischemia versus hypoxic conditions; (ii) determination of tissue injury and viability versus recovery of contractile function; and (iii) differences in the pre- and post-conditioning protocols applied.

The fact that the effect of conditioning interventions may cause a variable outcome depending on the protocol used, ranging from protection to damage, emphasizes the need for the clarification of these issues in laboratory studies before their use in man. This view would be supported by the absence of clinical benefit seen in the recently published DANAMI-3-iPOST clinical trial [22] in which routine IPostC during primary angioplasty failed to reduce a composite outcome of death and hospitalization for heart failure in patients with an acute myocardial infarction. Furthermore, in the ERICCA study [23], in which 1612 patients underwent elective on pump coronary artery bypass grafting with or without valve surgery and without standardization of the anesthetic regimen, remote IPreC using transient-arm I/R did not improve clinical outcomes. Similarly, the RIPHeart study [24], in which 1403 patients undergoing elective cardiac surgery and using propofol in anesthesia were randomized to receive either upper-limb remote IPreC or to become sham-control, the conditioning treatment did not show a relevant benefit. Although these recent large multicenter trials using remote IPreC have proven unsuccessful in cardiac surgery, it is necessary to acknowledge that the use of different anesthetic protocols and the presence of various underlying risk factors could have had an influence on the endogenous cardioprotective mechanisms with impairment of the response to the conditioning protocols. Furthermore, the use of cardioplegic solutions and other cardioprotective agents might have also played a role.

A limitation of our studies is the use of atrial right appendage as it may not fully represent the response of ventricular myocardium. However, we [25] and others [26] have observed that the right atrial and left ventricular myocardium have similar tolerance to ischemia and comparable response to protective interventions such as IPreC. Nonetheless, although the right atrial and left ventricular myocardium may also have similar response to IPostC, caution must be taken when extrapolating the present results to clinical conditions. Another potential limitation may be the number of right atrial appendage donors studied that, although it was sufficient to address the primary aim, it was insufficient to assess the role of the different clinical and cardiac conditions on the response of the myocardium to the use of IPreC and IPostC in combination. As shown in a previous study from our laboratory [10], the elucidation of these important issues would require well powered studies with larger number of right atrial donors.

Conclusion

A major contribution of our study is the demonstration of the lack of a uniform response to IPreC and IPostC and that the combined use of the two treatments improves protection although does not abolish the further myocardial injury that occurs in some instances in response to IPostC. Therefore, our results do not support the clinical use of IPreC and IPostC, either alone or in combination. Rather, additional basic research is needed to understand the underlying mechanisms induced by these treatments to better exploit their therapeutic potential. In this connection, manipulation of specific elements of the transduction cellular mechanisms could be a more effective way to afford protection and overcome the lack of responsiveness to IPreC and IPostC.

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Conflict of Interest

The authors report no conflict of interest.

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