Duration of the Reoxygenation Interval Applied before Ischemic Post-conditioning: Fine-Tuning the Protocol for Human Myocardium

Paula Soler-Ferrer1, Kelly Casós1, Maria L. Pérez1 and Manuel Galiñanes1,2*
1Reparative Therapy of the Heart, Vall d’Hebron Research Institute (VHIR), Barcelona, Spain
2Department of Cardiac Surgery, University Hospital Vall d’Hebron, Autonomous University of Barcelona (UAB), Barcelona, Spain

Corresponding author: Manuel Galiñanes, Consultant/Head “Reparative Therapy of the Heart”, Hospital Vall d’Hebron, Cardiac Surgery, Passeig Vall d’Hebron 119-129, Barcelona, Barcelona 08035, SPAIN, Tel: (+34) 932746160; Fax: (+34) 932746052; E-mail: manuel.galinanes@gmail.com

Received date: December 11, 2017; Accepted date: December 20, 2017; Published date: December 26, 2017

Copyright: ©2017 Soler-Ferrer P, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Ischemic post-conditioning (IPostC) is a cardioprotection strategy applied after prolonged ischemia. In an in vitro model of human myocardium we previously demonstrated that one cycle of 120 s of ischemia is the most protective; however the optimal duration of the reperfusion interval between prolonged ischemia and the application of IPostC have not been determined. To investigate the importance of this reperfusion interval, myocardial muscles from the right atrial appendage of 26 patients were subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced by four different reperfusion intervals (30, 60, 120 and 180 s) followed by 120 s of ischemia. Lactate dehydrogenase leakage and caspase 3 activity were measured to assess cell injury and apoptosis, respectively. The results showed that intervals of 120 and 180 s were more protective than those of 30 and 60 s, although protection was obtained in only approximately one-third of the patients. Importantly, the muscles from patients receiving nitric oxide donors as anti-angiinal agents were not protected by any of the IPostC protocols used. In conclusion, the present study demonstrates the importance of the duration of the reoxygenation interval before the application of IPostC, with 120 and 180 s conferring the greatest protection. This finding is relevant for the design of future studies on the clinical utility of IPostC and on the investigation of the underlying protective mechanisms.

Keywords: Ischemic post-conditioning; Human myocardium; Ischemic injury; Apoptosis

Introduction

Cardiovascular diseases remain the main cause of death and disability in the world [1]. In patients suffering myocardial infarction, the infarct size is a major determinant of ventricular remodeling and the most important determinant of heart failure [2]. Therefore, therapeutic efforts are aimed at limiting the infarct size, usually by early reperfusion through percutaneous coronary intervention or intravenous thrombolysis. Both therapies are effective in preventing post-infarction heart failure and improving survival [3,4]. However, reperfusion after prolonged ischemia also produces a paradoxical myocardial injury that may limit the efficacy of reperfusion therapies [5]. The detrimental effect of reperfusion injury can be counteracted by interventions such as ischemic post-conditioning (IPostC), which consists of brief repetitive coronary occlusions during the early reperfusion period. IPostC was first described by Zhao et al. in a dog model of myocardial infarction [6]. In this study, three cycles of 30 s of reperfusion/ischemia each after 60 min of ischemia followed by reperfusion reduced infarct size to a degree similar to ischemic preconditioning (IPreC), a phenomenon that renders the myocardium more resistant to an ischemic insult by the previous application of short periods of ischemia [7]. However, the results from other animal models and clinical studies on the efficacy of IPostC have been controversial, as benefits [8-10], no effect [11-13] and detrimental effects [14,15] have all been described. One reason for these variable results may be the use of different IPostC protocols. Using an in vitro model of ischemia/reoxygenation of human myocardium, our laboratory reported that the most effective IPostC protocol was one 120 s cycle of reperfusion/ischemia after 90 min of normothermic global ischemia [16]. However, the optimal time of the interval between the termination of prolonged ischemia and the application of the short ischemia of the IPostC protocol, a time when reperfusion injury is most likely, remains unclear. Hence, the aim of the present study was to investigate the most effective duration of the reoxygenation period within the IPostC protocol in the human myocardium.

Methods

The study was approved by the local ethics committee and each patient signed the consent to participate (ID-RTF065) in the study. Right atrial appendages were collected from 26 patients undergoing elective cardiac surgery, without any exclusion criteria other than emergency surgery. Demographic data, the presence of risk factors, and the treatment history of the patients were recorded.

Experimental preparation and study protocol

Right atrial appendages were obtained immediately before the patients underwent cardiopulmonary bypass. The tissues were transferred to the laboratory in Krebs Henseleit HEPES (pH 7.4) buffer [118 mM NaCl, 4.8 mM KCl, 27.2 mM NaHCO3, 1.2 mM MgCl2, 1.0 mM KH2PO4, 1.25 mM CaCl2, 10 mM glucose, 10 mM HEPES (Sigma-Aldrich, USA)] at 4°C. Connective and adipose tissues were discarded and the muscles were sliced to obtain 300-500 µm stions using a surgical skin-graft knife (Swann-Morton, Sheffield, England). The muscle slices were then equilibrated for 40 min in Henseleit...
HEPES buffer (pH 7.4), oxygenated by bubbling the medium with 95% O2/5% CO2, and maintained at 37°C throughout the experiments. Simulated ischemia was induced by bubbling the medium with 95% N2/5% CO2 (pH 6.8) and replacing d-glucose by 2-deoxy-d-glucose, as described previously [17].

The experimental protocol is depicted in Figure 1. Briefly, samples were subjected to 90 min of normothermic ischemia followed by 120 min of reperfusion. One subset of muscles was subjected to ischemia/reperfusion (I/R) alone. The other muscles were subjected to a single cycle of IPostC in which the duration of ischemia was fixed at 120 s, a time previously shown to be the most effective IPostC protocol in our laboratory [16], and preceded by different reoxygenation intervals: 30, 60, 120, or 180 s. Samples not subjected to ischemia and perfused aerobically during the entire experimental period served as aerobic controls (AC).

Assessment of tissue injury

Tissue injury was assessed by measuring the leakage of lactate dehydrogenase (LDH) into the incubation medium during the 120 min reoxygenation period using a kinetic method based on the reduction of NADP to NADPH. The absorbance of the samples was read at 340 nm using a Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, USA) and the results were expressed as arbitrary units (AU)/g wet tissue.

Assessment of apoptosis

Tissue apoptosis was determined at the end of the experiment by measuring caspase 3 (C3) activity using a colorimetric method according to the suppliers description (BioVision, Milpitas, USA). The tissues were homogenized (Omni International, Kennesaw, USA) and the absorbance was measured at 405 nm using a Multiskan FC plate reader (Thermo Fisher Scientific). The results were expressed as ng active C3/mg protein.

Statistical analyses

Continuous variables were expressed as the means ± standard error of the mean and compared using the Wilcoxon test. Pearson's correlation was used to evaluate the association between myocardial protection and ischemic injury. Contingency tables were used to study the effect of donor-related factors, including concomitant cardiac pathologies, associated co-morbidities, and medical treatment. All statistical analyses were performed using SPSS 20 and GraphPad Prism 6. A p value<0.05 was considered to be statistically significant.

Results

Table 1 shows the demographic data, the type of heart disease and the associated comorbid conditions of donor patients.

| Sex       | Female | Male |
|-----------|--------|------|
|           | 9 (34.6%) | 17 (65.4%) |
| Age >60   | 14 (53.8%) |      |
| Age ≤ 60  | 12 (46.2%) |      |
| CAD       | 12 (46.2%) |      |
| AVD       | 16 (61.5%) |      |
| MVD       | 10 (38.5%) |      |
| TVD       | 7 (26.9%) |      |
| Ostium sundum ASD | 1 (3.8%) |      |
| LVEF >40% | 26 (100%) |      |
| LVEF ≤ 40% | 0 (0%) |      |
| AF        | 4 (15.4%) |      |
| Obesity   | 7 (26.9%) |      |
| Dyslipidemia | 11 (42.3%) |      |
| Hypertension | 19 (73.1%) |      |
| Diabetes  | 7 (26.9%) |      |
| Smoker    | 5 (19.2%) |      |

Figure 2A shows that the LDH leakage mean values of muscles subjected to reoxygenation intervals of 120 and 180 s before the application of IPostC were not significantly different from the values seen with muscle subjected to I/R alone; however, LDH values were significantly more elevated in the groups subjected to reoxygenation intervals of 30 and 60 s, this indicating an increase in tissue damage. Figure 2B shows that similar results were obtained for C3 activity with the shortest periods of reoxygenation affording the highest levels of apoptosis.
Figure 2A: Effect of the duration of the reoxygenation interval before ischemic postconditioning (IPostC) on lactate dehydrogenase (LDH) leakage of human right atrial myocardium (N=26 donors) subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced using 30, 60, 120, and 180 s intervals of reoxygenation prior to 120 s of ischemia. *p<0.05 vs. the ischemia/reoxygenation (I/R) alone group.

Figure 2B: Effect of the duration of the reoxygenation interval before ischemic postconditioning (IPostC) on caspase 3 activity of human right atrial myocardium (N=26 donors) subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced using 30, 60, 120, and 180 s intervals of reoxygenation prior to 120 s of ischemia. *p<0.05 vs. the ischemia/reoxygenation (I/R) alone group.

Figures 3A-3D shows the individual results for LDH leakage in all the study groups. They demonstrate that IPostC with a reoxygenation interval of 60, 120 and 180 s reduced LDH leakage below the mean I/R alone group value (eg: protective) in approximately one-third of the muscles. However, LDH leakage was reduced in only 10% of the muscles treated with 30 s reoxygenation interval.

Figure 3A: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 30 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.

Figure 3B: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 60 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.

Figure 3C: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 120 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.
An analysis of the influence of concomitant cardiac pathologies, associated comorbid conditions, and the medical treatments received by the muscle donors on the response to IPostC revealed a significant negative relationship between IPostC and patients receiving nitric oxide (NO) donors (data not shown). In none of these patients the myocardium was protected by the most effective IPostC protocol.

**Discussion**

Since the first report, in 2003, that IPostC induced by three 30 s cycles of reperfusion and ischemia can reduce infarct size by 44% in anesthetized open-chest dogs with a left anterior descending artery occluded for 60 min [6], the beneficial effect of this type of intervention has been demonstrated in several animal models [18] and in man [19-21]. However, other investigators have reported no benefit [11-13] or even increasing of the ischemic damage [14,15]. It is worth noting that in the induction of IPostC the number of cycles and their duration varied and none of the studies examined the importance of the duration of the reoxygenation interval between the ischemic insult and IPostC application. Reperfusion injury, which includes calcium overload and the production of oxygen free radicals, occurs during the first few minutes of reperfusion [6,22-24], and therefore it is of critical importance to determine what is the optimal reoxygenation interval for the application of IPostC. Our study is the first to demonstrate that the human myocardium is better protected by an IPostC protocol with a 120 or 180 s reoxygenation interval than when an interval of 30 or 60 s is used. It also confirms our previous finding [16] that despite this only approximately one-third of the human myocardium samples obtained from patients undergoing cardiac surgery can be protected by the best IPostC protocol.

The present study also showed that the mechanisms underlying the beneficial effect of IPostC include a decrease in tissue injury, as assessed by LDH leakage, and a reduction of apoptosis, as determined by C3 activity. These findings in human myocardium are supported by a study performed in primary cultured neonatal rat cardiomyocytes exposed to 3 h of hypoxia followed by 6 h of reoxygenation and then to three 5 min cycles of reoxygenation/hypoxia [25]. The decrease in cardiomyocyte apoptosis was shown to be mediated by reductions in the release of tumor necrosis factor-α and in C3 expression, via inhibition of the JNKs/p-38 signaling pathway. In a study of patients with acute myocardial infarction undergoing percutaneous coronary intervention, IPostC also resulted in a reduction of apoptosis, as measured by the serum concentration of sFas and sFas [26].

Importantly, none of the myocardial slices from patients treated with NO donors was protected by IPostC. Fakete et al. [27] also reported the loss of protection by IPostC in isolated rat hearts in which nitrate tolerance was induced by the administration of nitroglycerin 3 days before the ischemic insult. Similar findings were obtained in a rabbit model, in that study, nitrate tolerance abolished the protection afforded by IPreC [28,29]. Together, these results highlight the important role of NO in the intracellular signal transduction mechanism of IPreC and IPostC [30,31].

In conclusion, our study showed that the duration of the reoxygenation interval before the application of IPostC is important in optimizing the protection of human myocardium against I/R-induced injury and that, in the *in vitro* model used, intervals of 120 and 180 s of reoxygenation confer the best protection. These findings do not support a wide clinical application of IPostC until the most effective protocol is fully defined in clinical settings and the patients that can benefit from the treatment are identified. This approach is justified by the results recently published of the DANAMI-3-IPOST clinical trial [13] in which routine IPostC during primary angioplasty failed to reduce a composite outcome of death and hospitalization for heart failure in 1234 patients with an acute myocardial infarction. It is clear that further laboratory research will be required for a better understand of the mechanism of protection by IPostC that would help to refine the much needed interventions of effective therapies at the time of reperfusion.

**Funding**

The current study was supported by the Instituto de Salud Carlos III (FIS) [grant number12/00119].

**Acknowledgments**

We are grateful to the members of the Cardiac Surgery Department of University Hospital, Vall d’Hebron for providing myocardial atrial samples from patients who provided informed consent.

**References**

1. Hausenloy DJ, Yellon DM (2009) Preconditioning and postconditioning: Underlying mechanisms and clinical application. Atherosclerosis 204: 334-341.
2. Sutton MGSJ, Sharpe N (2000) Left ventricular remodeling after myocardial infarction pathophysiology and therapy. Circulation 101: 2981-2988.
3. Luna-Ortiz P, Torres JC, Pastelin G, Martinez-Rosas M (2011) Myocardial postconditioning: anesthetic considerations. Arch Cardiol Mex 81: 33-46.
4. Ratcliffe AT, Pepper C (2008) Thrombolysis or primary angioplasty? Reperfusion therapy for myocardial infarction in the UK. Postgrad Med J 84: 73-77.
5. Figueredo VM, Diamond I, Zhou HZ, Camacho SA (1999) Chronic dipyridamole therapy produces sustained protection against cardiac ischemia-reperfusion injury. Am Physiol Soc 277: 2091-2097.
6. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, et al. (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol 285: H579-H588.
7. Tomai F, Crea F, Chiariello L, Gioxéffé PA (1999) Ischemic preconditioning in humans models, mediators, and clinical relevance. Circulation 100: 559-563.
8. Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, et al. (2005) Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. Am J Physiol Heart Circ Physiol 289: 1618-1626.
9. Kin H, Zatta AJ, Loiye MT, Amerson BS, Halkos ME, et al. (2005) Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res 67: 124-133.
10. Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, et al. (2005) Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca2+ overload. Am J Physiol Heart Circ Physiol 288: 1900-1908.
11. Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P (2007) Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovasc Res 75: 168-177.
12. Couvreur N, Lucats L, Tissier R, Bize A, Berdeaux A, et al. (2006) Differential effects of postconditioning on myocardial stunning and infarction: a study in conscious dogs and anesthetized rabbits. Am J Physiol Heart Circ Physiol 291: 1345-1350.
13. Engstrom T, Kelbaek H, Helqvist S, Hofsten DE, Klovgaard L, et al. (2017) Effect of ischemic postconditioning during primary percutaneous coronary intervention for patients with ST-segment elevation myocardial infarction: a randomized clinical trial. JAMA Cardiol 2: 490-497.
14. Schwartz LM, Lagrampa CJ (2005) Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischemia-reperfusion injury in pigs. Am J Physiol Heart Circ Physiol 290: 1011-1018.
15. Dow J, Klener RA (2007) Postconditioning does not reduce myocardial infarct size in an in vivo regional ischemia rodent model. J Cardiovasc Pharmacol Ther 12: 153-163.
16. Casós K, Pérez ML, Blasco-Lucas A, Ferrer-Curriu G, Gracia-Baena JM, et al. (2015) Ischemic postconditioning of the isolated human myocardium: Role of the applied protocol. Int J Cardiol Heart Vasc 8: 55-61.
17. Casós K, Ferrer-Curriu G, Soler-Ferrer P, Pernamney E, Blasco-Lucas A, et al. (2017) Response of the human myocardium to ischemic injury and preconditioning: The role of cardiac and comorbid conditions, medical treatment, and basal redox status. PLoS One 12: e0174588.
18. Skyschally A, van Caster P, Blidromitis EK, Schulz R, Kremastinos DT, et al. (2009) Ischemic postconditioning: experimental models and protocol algorithms. Basic Res Cardiol 104: 469-483.
19. Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, et al. (2007) Postconditioning protects human atrial muscle through the activation of the RISK pathway. Basic Res Cardiol 102: 453-459.
20. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, et al. (2005) Postconditioning the Human Heart. Circulation 112: 2143-2148.
21. Fan Q, Yang XC, Liu Y, Wang LF, Liu SH, et al. (2011) Postconditioning attenuates myocardial injury by reducing nitro-oxidative stress in vivo in rats and in humans. Clin Sci 120: 251-261.
22. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, et al. (2004) Multiple Brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 44: 1103-1110.
23. Huhn R, Heinen A, Weber NC, Schlack W, Preckel B, et al. (2010) Ischaemic and morphine-induced post-conditioning: impact of mKCa channels. Br J Anaesth 105: 589-595.
24. Jin C, Wu J, Watanabe M, Okada T, Isakiti T (2012) Mitochondrial K+ channels are involved in ischemic postconditioning in rat hearts. J Physiol Sci 62: 325-332.
25. He-Ying S, Wang NP, Halkos M, Kerendi F, Kin H, et al. (2006) Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways. Apoptosis 11: 1583-1593.
26. Zhao WS, Xu L, Wang LF, Zhang L, Zhang Zy, et al. (2009) A 60-s postconditioning protocol by percutaneous coronary intervention inhibits myocardial apoptosis in patients with acute myocardial infarction. Apoptosis 14: 1204-1211.
27. Fekete V, Murlasits Z, Ayap E, Bencsi P, Sárközy M, et al. (2013) Myocardial postconditioning is lost in vascular nitrate tolerance. J Cardiovasc Pharmacol 62: 298-303.
28. Szilvassy Z, Ferdinandy P, Bor P, Jakab I, Szilvassy J, et al. (1994) Loss of postconditioning in rabbits with vascular tolerance to nitroglycerin. Br J Pharmacol 112: 999-1001.
29. Szilvassy Z, Ferdinandy P, Nagy I, Jakab I, Koltai M (1997) The effect of continuous versus intermittent treatment with transdermal nitroglycerin on pacing-induced preconditioning in conscious rabbits. Br J Pharmacol 121: 491-496.
30. Barua A, Standen NB, Galifianes M (2010) Modulation of the nitric oxide metabolism overcomes the unresponsiveness of the diabetic human myocardium to protection against ischemic injury. J Surg Res 171: 452-456.
31. Hu L, Wang J, Zhu H, Wu X, Zhou L, et al. (2016) Ischemic postconditioning protects the heart against ischemia–reperfusion injury via neuronal nitric oxide synthase in the sarcoplasmic reticulum and mitochondria. Cell Death Dis 7: e2222.